

nature

8 September 1977

When science turns up the unpalatable

THE Presidential Address of Sir Andrew Huxley to the British Association for the Advancement of Science, meeting in Aston, has been hyperbolically described in the press as "the first shot in what could become one of the most crucial and vitriolic scientific battles of the century". It certainly is an unusual, even courageous address.

His line of arguing is this. He asserts that "Science as a whole—the scientific approach to questions of all kinds—has come increasingly under attack . . . it is repeatedly suggested that the speed of scientific discovery should be slowed, and that scientists ought to suppress discoveries that seem capable of being used to the detriment of humanity". In a lengthy aside, he then acknowledges that scientists themselves often rely too much on grand unifying principles which inhibit their collection of evidence. But in a broader context too the collection of scientific evidence may be inhibited if it seems likely to touch on some particularly sensitive nerve in ordinary human affairs. Resistance, he claims, may be based on "Authority" or on the fear of the consequences of accepting the conclusion. Not for nothing does a Huxley hark back to the 1860s and the debate on this very matter occasioned by the publication of *Origin of Species*.

He asks if any topic stirs similar emotions these days. And he sees many features in common between the evolution debate of the last century and the current debate on the extent to which human ability is inherited, although he points out that the analogy would be better had Darwin gone public twenty years earlier when his case would have been weaker, but certainly not hopeless. The resistance these days to research in the field of the heritability of human ability comes from a feeling that our ethics may be undermined because the existence of substantial inherited differences would lead to unjust treatment of the less-well endowed, and that discoveries of below average ability would damage self-respect.

More sinister than anything in the evolution controversy, says Sir Andrew, is that there are actually scientists who regard the assumption of equal inherited ability as "something which does not require experimental evidence to establish it and which it is positively wicked to question because the conclusion might disagree

with their social and political preconceptions". Thus when someone (William Shockley) had the "courage to suggest systematic and scientific investigation" he was repeatedly turned down by the National Academy of Science. Sir Andrew accepts that at the time Shockley was advocating eugenic measures unacceptable to public opinion, but thinks the main reason for refusals was fear that it would be represented as a "commitment to an illiberal point of view unfavourable to American Negroes". He feels that such behaviour is an unjustifiable obstacle to human enquiry and impedes or distorts the advance of science.

Freedom for scientists to investigate whatever they want may be a valid rallying point in some circumstances and under some tyrannies. But scientists are already under some external constraints on their freedom, for ethical and financial reasons, and it is clearly not possible simply to say that everything that is observable and measurable ought, in the name of science, to be observed and measured. The question then revolves around the motives for doing research into heritability. There are, of course, two extremes—those who believe that the world should know that the less well-endowed are going to stay that way and those who believe that the whole thing is both scientifically unsound and an affront to certain people, and should be stopped forthwith.

Is there middle ground? If there is, and many intelligent and thoughtful geneticists believe there is, it needs staking out with immense care. Those who propose that research be stopped have some very human motives behind them, and it is no answer simply to wave freedom of scientific enquiry in their faces. Questions are bound to be asked about motives for doing more research, especially as there is little doubt that inheritance does play at least some and maybe a very significant part in the acquisition of ability. Some who want to do more only want to do so to rub in assumed inferiorities. What Sir Andrew should have done is take his argument further and set forth reasons why more research at this time, with its close proximity to sensitive issues, is desirable. Such a case can probably be made out but it would need a rather more spirited explanation of why we are better off with the knowledge than without it. □



La Soufrière, volcanology and forecasting

Last year Haroun Tazieff, of the Laboratoire de Volcanologie, Centre de Faibles Radioactivités, Gif-sur-Yvette, France, had to leave his post as director of the Service Volcanologique at the Institute of Physics of the Globe over the eruption of La Soufrière. Below, his account of the problems

IN THE past six years at least four erroneous volcanological diagnoses, based either on wrong interpretations of actual facts or on deliberately false data, have induced governmental authorities in four countries to take unnecessary and expensive measures. Together they show that volcanologists may sometimes be confronted with quite serious socio-economic consequences of their own forecasts.

● The first case was in 1970 in Italy's Neapolitan area. A submarine eruption was claimed to have burst out offshore of the city of Pozzuoli, approximately 2 km west of the well-known Solfatara fumarolic field. After the mass-media told of possible explosions, ash-falls and sea-waves, the Army and the *carabinieri* (police) helped the frightened population to evacuate the city. The claimed symptoms were an upheaval of the ground in the whole area, which had been pushed up about one metre in a "very short while"; extremely shallow earthquakes, located 1 to 2 km offshore; a conspicuous temperature-rise of the Solfatara's fumaroles; and the outflow of hot fluids on the bay's floor, inferred from dead boiled fish fishermen took in their nets.

The last three items were subsequently proved totally false by a team of six scientists led by myself, and I disclosed the fact at a press conference. On the other hand, the upheaval, the so-called brady-seism of Pozzuoli, had not been faster than usually noticed over the centuries during which up-and-down level variations of that peculiar area had been classically observed. Eventually it was stated that deliberately wrong data had been produced by the scientist in charge of the geophysical institute of Naples. This was connected to a construction operation in which high-rank people were involved. The evacuation of the inhabitants was proved to be expensive and useless.

● The second case happened during the 1972 eruption of St Vincent's Soufrière in the West Indies. Notwithstanding the clearly expressed and reassuring opinion of Dr J. Tomblin, the volcanologist in charge, an alarming cable from a scientist some thousand kilometres away was taken into consideration. This 'televolcanologist' had deduced his frightening conclusions from earth-satellite imagery and the whole population was consequently moved away. Here too it proved both expensive and useless.

● The third case happened in 1973 during the last days of the Heimaey eruption. Persuaded by a somewhat inexperienced foreign volcanologist, Icelandic authorities agreed to use fire-boats to sprinkle water on a tongue of the thick lava flows which over several weeks had progressed at a distressing speed towards the harbour entrance. No arguments could prevent the exercise: not even the evidence that the Atlantic ocean itself, with all its water, had not been able to stop the main part of the flows which had crawled over the sea-floor for two months.

● The fourth error occurred in 1976 during the eruption of La Soufrière in Guadelope in the West Indies. Here too, in spite of the firm statement of the volcanologist in charge that no danger lay ahead, the authorities followed the alarmist opinion expressed first by a petrographer and then

by a geochemist, both of whom lacked experience of eruptive phenomena. Consequently 73,600 people were evacuated on 16 August and were kept away from their homes and jobs for 3½ months. It proved very expensive, dramatic for the population, and totally useless.

A volcanologist is actually as responsible for his diagnoses as is a physician—even more so, because of the number of people involved and because the costs are usually far bigger. This implies that some sort of deontological code, similar to the medical one, should exist for practising volcanologists, and that illegal practice should be prohibited in this field just as it is for medicine. Whatever their field, scientists cannot remain detached from the eventual effects of their work on the everyday life of other people.

The easiest course for the authorities is naturally to choose, amongst various scientific counsels, the most pessimistic if not necessarily the best one. Their responsibility is then shielded against the worst, and if the catastrophe eventually does not occur the only reproaches they suffer are minor ones that are swiftly forgotten. To express a volcanological forecast is always an awkward business. Like civil administrators, many a volcanologist will naturally tend to be pessimistic rather than optimistic, if only to avoid exposing people to some danger he would have disregarded; he would want to avoid being accused if his own optimistic forecast proved unfounded. Though understandable enough, such an attitude is not in accord with the deontology code; a deliberately pessimistic physician is not necessarily considered a good medical doctor.

But some points seem clear. First of all, no volcanologist should state any opinion if his knowledge of eruptive phenomena is below a minimum level. Secondly, the consultant volcanologist should express his scientific opinion without altering it in either an optimistic or pessimistic way. An honest volcanologist, whatever his experience, all too frequently will not be able to describe much more than his incapacity to tell what will follow next. Nevertheless, a valuable forecast is sometimes possible and our own experience shows examples of it.

It actually happened in 1976 at the Guadelope Soufrière, when a rather unusually flat and valid conclusion seemed evident to any experienced volcanologist: absolutely no risk existed that the volcanic event which the whole Caribbean population most feared—*nuées ardentes* (glowing avalanches) of the Mt Pelée type—could happen at all in the near future. I delivered this view, while several other scientists claimed the opposite. But it was obvious that the eruptive events, which were started during the autumn of 1975 by a volcano-seismic crisis, had then passed to phreatic kinds of outbursts in which no fresh magma at all was involved, and would not develop into *nuées ardentes*. The French government nevertheless chose not to listen to this plain argument, asked geologists unskilled in volcanology and proceeded to evacuate the entire population of 73,600 people. The whole issue developed in phases.

● The seismic activity of the volcano started in October 1975 when the seismographic array of the Soufrière Observatory began recording swarms of microearthquakes. For several months, these swarms increased in both number and intensity; the first felt earthquake occurred in late March 1976. At this point several scientists expressed anxiety, more because of the high number of recorded microearthquakes than because of their actual energy. None amongst them had any experience in eruptive phenomena.

The opposite opinion was based upon the two following facts. First, far more frightening volcano-seismic crises had previously been observed in the Caribbean, and many more

still in Japan, where the most earnest attempts in the world have been made in volcanological forecasting. At the Omuro volcano in 1930, for example, 4,880 shocks located between 2–7 km below the surface were recorded in less than three months; at the Hakone volcano in 1959–60, the foci of the recorded earthquakes were located 0.8–5 km below the surface, but on the basis of the complete lack of any B-type earthquake shallower than 0.8, Minamaki predicted that no volcanic eruption would happen and he was right. In the Caribbean, two volcano-seismic crises occurred at Montserrat island in 1897–98 and 1933–36, and are well known to volcanologists; neither of these well-studied crises finished with volcanic eruption. It therefore made sense not to be unduly alarmed by the 1976 seismic events of the Soufrière.

Secondly, the focal depths of the earthquakes during the Soufrière crisis were located at 2–6 km, depths quite similar to those which had characterised the Hakone and the Omuro crises. This meant that many months, or more probably years, should be necessary for the Soufrière viscous magma to reach the surface, for any magma is supposed to be far slower than its own lavas, and all the Soufrière lavas had obviously been comparatively viscous and slow. Experience has shown that most frequently the average speed of lava flows of the same kind as those of the Soufrière varies between less than 1 and about 100 cm h⁻¹. From a spot located below 6 km down, the corresponding feeding magma should therefore require several years to reach the surface. If any factors had been overlooked, or if the magma velocity would drastically increase, several months seemed a minimum for it actually to become eruptive. Consequently one could quite confidently speak of a period of several weeks, at least, before the start of any magmatic event.

To the objection that a body of molten (and therefore not seismic) magma could be stored *above* the uppermost foci, that is, between the surface and the 2 km depth—meaning that a hypothetical reservoir was located within the 1,400 m thick volcanic heap itself—the answer was that both the temperatures measured close by in the geothermal field of Bouillante (70 °C near the surface and 240 °C 350 m down) and the gas composition of the Soufrière fumaroles (in vol % : CO₂, 90–93%; H₂S, 0.6–1%; CH₄, 0.5%; H₂, 0.5–1.2%) proved such a hypothesis to be quite improbable.

● On 8 July 1976 the eruption started its second, actually eruptive, phase when so-called ‘ash’ outbursts were superimposed on the almost continuously increasing volcano-seismic activity. This second phase lasted eight months, during which 20 phreatic eruptions occurred, the last one on 1 March 1977. Each outburst lasted only a few minutes (less than 20) and expelled steam and water droplets mixed with some volcanic gases laden with ejecta. These ejecta, ‘ashes’, sands and blocks, were exclusively composed of old rock material, with no trace of any fresh magmatic material. The first of these phreatic eruptions started noiselessly and propelled a dark mushroom hundreds of metres above the top of the mountain. This plume was then driven south-westwards by the wind. From the volcano windwards, the sky became totally dark, and a thick rain of small lapilli and dust started, which lasted for about 20 minutes. Several thousand people fled as soon as the darkness cleared up.

The scientists called by the authorities were divided. Some of them felt afraid, firstly because they feared that these ‘ash explosions’ could lead either to *nuées ardentes* or to mud-flows, or both. The opposite opinion was that these risks did not exist because this eruption obviously belonged to the phreatic kind: gas analyses, measured steam temperatures, as well as examination of ejecta showed that no fresh hot magma or lava were involved and that, consequently, no *nuées ardentes* could be expected. On the other hand, as in phreatic eruptions, the

very first outburst usually is the strongest one, and as the 8 July one had generated only a small mud-flow, it was to be supposed that further lava hazards would be minor. In spite of an apparent tendency to prefer pessimistic diagnosis to reassuring ones, the government authorities were consequently prevented from evacuating people.

The second phreatic outburst occurred on 24 July. Asked by the *prefet* and having obtained the data from and opinion of my collaborators, now permanently monitoring the fluid and solid exhalation of the volcano, I concluded that there was no more danger ahead than two weeks before.

● The third outburst happened on 9 August. I was in the Ecuadorian Andes involved in a volcanological expedition with two of my collaborators, but our three colleagues were still on La Soufrière, mainly to monitor the chemical and physical evolution of the gas phase, which may be considered as a good indicator of the eruptive evolution itself. These skilled volcanologists declared that no threatening change was to be feared in the eruptive events. This was because of the following facts: first, the gas composition had not changed. The proportion of CO to CO₂ was about 10⁻³–10⁻⁴, and of H₂S to SO₂ was about 10; in addition, the focal depth of the earthquake had not moved, no trace of any magmatic activity was detectable anywhere, either in the erupting area or on the lower slopes of the volcano, and old rocky material *exclusively* was to be found around the eruptive vents.

Disregarding these volcanologists’ opinions, the civil authorities nevertheless called from France two geologists without any experience in the field of eruptive activity. The first decided to have the whole population immediately evacuated; the second decided to enforce the precautions and maintain them for several months on.

Their diagnoses were based, first, on the assumption that the seismo-volcanic crisis—up to 1,257 shocks, half a dozen of which had been slightly felt, in one day—had reached such a climax that nothing but a catastrophe could proceed, and secondly on the claimed presence of fresh volcanic glass in the erupted ‘ashes’, the meaning of which was the imminence of *nuées ardentes*. Professor C. Allègre, on 5 September 1976, produced an official report in which he stated that 50–60% of fresh volcanic glass was present in the erupted ash. He was later compelled to admit that actually no trace of fresh material had ever been detected.

According to *Le Guern* (in press) the twenty outbursts which occurred between the first (8 July 1976) and the last one (1 March 1977), as well as the more or less continuous emission observed at the active vents throughout the same period, have poured out a total output of the order of 6–10 × 10⁶ tons of steam and 1.5–2 × 10⁶ tons of ‘ashes’ and blocks. According to the same author, a surface less than 1 km long and 300 m wide received a total ‘ash’ fall 300 m thick for the whole nine-months-long event. The closest town, Matouba, some 3.5 km west (windwards) from the craters, got a total ‘ash’ fall of 5–15 mm according to the spots. The closest city, St-Claude, 4.5 km west-south-west from the erupting vents, got a total 2–5 mm thickness of ‘ash’. The longest mud-flow, which resulted from the first rain eastwards over less than 3 km.

These figures show how modest was the scale of this eruption. The above mentioned criteria and their interpretation explain how our so-called ‘optimistic’ diagnosis had been attained. The main lesson of this unfortunate experience is perhaps to confirm the absolute necessity for a good volcanological interpretation of all the available, geological, geophysical, chemical and phenomenological data before expressing any forecast. Volcanologists, just as medical doctors, should be responsible, skilled, experienced, different specialists closely co-operating with each other. And they should keep as cool as a cucumber. □

Voyage to the outer planets

Both NASA's Voyager spacecraft are heading for the outer planets. **Stuart Sharrock** describes the journey they will make

WHEN the outer planets were last aligned so that a spacecraft could visit each in turn on a single voyage, Thomas Jefferson was President of the United States. Understandably none took advantage of that opportunity. But in the 1980's, the opportunity to fly past each outer planet will occur again and if it is not seized then it will be lost for several generations.

NASA's original plan to take advantage of the 1980's alignment consisted of sending two spacecraft on a visit to every planet from Jupiter to Pluto. Nicknamed the 'Grand Tour', that project soon fell victim to budget cuts and the current Voyager mission was developed as something of a consolation prize. The Voyager mission, however, is itself ambitious enough. Its two spacecraft, launched on 20 August and 5 September, are intended to scrutinise Jupiter, Saturn and their moons, with an option for one to continue to Uranus and Neptune.

A mission to the outer planets is a radically new adventure. The inner planets, Mercury, Venus, Earth and Mars are rocky worlds which have been intensively studied. They seem to bear little resemblance to the gaseous outer planets that dominate the solar system. But very little is known about Jupiter and Saturn and next to nothing about Uranus and Neptune. Only two spacecraft have ever reported from the region beyond Mars; Pioneers 10 and 11 flew past Jupiter and returned intriguing but inconclusive data. Pioneer 11 is expected to reach Saturn in 1979.

The two Voyager spacecraft will reach Jupiter in 1979. More massive than all the other planets put together, Jupiter is an enormous ball of gas at the centre of a miniature solar system of its own. Four of its thirteen known moons are as large as some planets and Voyager will take a close look at five of them, concentrating on Io with its associated strong concentration of charged particles which appear to modulate the powerful radio bursts from Jupiter itself.

By visiting Jupiter, Voyager will be able to halve the journey time to Saturn. It will take advantage of the increase in velocity provided by Jupiter's gravitational field and should make the flight to Saturn in just over three years instead of the normal six. This 'gravitational assist' technique may be used again at Saturn to sling one of the spacecraft to a rendezvous with distant Uranus in 1986.

The spectacular rings around Saturn will be only one object for investigation. Like Jupiter, Saturn emits more energy than it receives and it has an extensive system of moons. Voyager will visit seven of the ten moons and will take particular interest in Titan, a moon larger than the planet Mercury and with an atmosphere. Titan is thought to be a far better place than Mars to search for evidence of extra-terrestrial life; the theory that life on Earth developed from a primitive reducing atmosphere implies early conditions on Earth similar to those which now exist on Titan.

Jupiter, Saturn and Titan are the prime objectives of the mission. If the first spacecraft, Voyager 1—which takes a faster route to Jupiter and was therefore the second to be launched—fails to complete its investigation of Titan, Voyager 2 will be redirected to do the job again. Only if Voyager 1 is successful will Voyager 2 fly directly towards Uranus and eventually, after a 13-year flight, to Neptune.

Special design features

Even if the mission does not extend beyond Saturn, its lifetime will be long and the distance travelled vast compared to previous missions. To cope with these conditions, the Voyager spacecraft require special design features. The familiar arrays of solar panels are missing as the energy received from the Sun as far away as Jupiter is only 4% of that at the Earth; at Saturn it is down to 1% and at Neptune 0.1%. Instead, radioisotope thermoelectric generators, which should last the journey to Neptune,

supply power to the spacecraft. The power requirements are great because signals have to be sent across such large distances. The spacecraft will be the first to communicate via the high frequency X-band channel, allowing high data rates which are essential at various stages of the mission.

The 11 instruments on board include television cameras, infrared and ultra-violet spectrometers, charged particle detectors, magnetometer and plasma wave detectors and, for the first time on an interplanetary probe, radio wave detectors. All experiments have to operate at liquid nitrogen temperatures and because of the length and complexity of the mission almost everything that could fail has a back-up system—including the two spacecraft themselves which are identical.

With such a complex mission the chance of something going wrong is high, but Voyager has already had more than its fair share of troubles. Very near 20 August, the launch date of the first spacecraft (designated Voyager 2), computer problems were discovered. A rapid switch of the two spacecraft meant that Voyager 2 could be launched on time and that Voyager 1 which was due for launch on 1 September would be delayed by a few days. But the first launch was rough. Computer failure occurred again, there were apparent problems with gyroscopic stabilisation and the boom containing the television cameras did not deploy fully. There were even speculations that the spacecraft had been struck by its own discarded rocket engine. The problems were overcome but inevitably the mission has already been compared with Mariner 10 which had to be nursed continually during its 17 month trip to Venus. The outlook for Voyager 1, though, is brighter. According to NASA it went up on schedule and with no hitches on 5 September. □

Voyager mission highlights

Date	Event	Range (km)	Resolution (km)	Spacecraft
20 August, 1977	Launch			Voyager 2
5 September, 1977	Launch			Voyager 1
15 December, 1978	Start Jupiter imaging	80×10^6	1.5×10^3	Voyager 1
5 March, 1979	Closest approach Jupiter	278×10^3	6	
5 March, 1979	Io	22×10^3	0.5	
April, 1979	Conclude Jupiter imaging			
20 April, 1979	Start Jupiter imaging	75×10^6	1.5×10^3	Voyager 2
10 July 1979	Closest approach Jupiter	643×10^3	13	
August, 1979	Conclude Jupiter imaging			
August, 1980	Start Saturn imaging	100×10^6	2×10^3	Voyager 1
11 November, 1980	Titan	4×10^3	0.5	
12 November, 1980	Closest approach Saturn	138×10^3	3	
January, 1981	Conclude Saturn imaging			
June, 1981	Start Saturn imaging	100×10^6	2×10^3	Voyager 2
27 August, 1981	Closest approach Saturn	102×10^3	2	
27 August, 1981	Edge Saturn rings	38×10^3	0.8	
October, 1981	Conclude Saturn imaging			Voyager 1 successful at Titan)
January, 1986	Uranus encounter			
September, 1989	Neptune encounter			

USA

Action on antibiotics

Colin Norman reports from Washington on the Food and Drug Administration's (FDA) recent action to ban the use of some antibiotics in animal feeds

AFTER more than a decade of controversy and several years of vacillation, the Food and Drug Administration (FDA) last week took the first formal step towards removing antibiotics from animal feeds, a step long urged by some scientists but bitterly resisted by the agricultural and pharmaceutical industries. The FDA issued a regulation which, provided it survives an expected slew of legal challenges, would prohibit the addition of small amounts of penicillin to premixed feeds for pigs and poultry. A similar regulation banning tetracyclines will follow soon, FDA announced.

Antibiotics have been added to animal feeds in the United States for 25 years, following a discovery that they help promote growth and hence shorten the road to the slaughterhouse. The practice has become so common that, according to figures collected by FDA, some 48.6% of all antibiotics produced in 1975 were added to animal feeds. In the mid-1960's, however, some scientists began to warn that such widespread use of antibiotics may be contributing to the growing problem of antibiotic resistance among microbes which cause human and animal diseases—resistance which is seriously compromising therapeutic uses of some drugs.

One concern is that bacteria which cause disease in animals will become resistant to antibiotics, thus making the disease more difficult to treat. Another concern is that the resistant bacteria may be transferred from animals to humans either directly or through meat, milk or eggs. An added dimension to the concern is the fact, which is now widely established, that antibiotic resistance can be passed from one strain of bacteria to another—for example, an animal bacterium which becomes resistant to an antibiotic may pass its resistance to a human pathogen through a chance encounter. (Antibiotic resistance is largely determined by so-called R-plasmids, tiny rings of DNA which sit inside a bacterial cell and are reproduced each time the bacterium divides).

Those concerns led the British government in 1968 to establish a special committee, under the chairmanship of Professor Sir Michael Swann,

to review the problem. The Swann Committee concluded "the administration of antibiotics to farm livestock, particularly at the sub-therapeutic levels, poses certain hazards to human and animal health", and it recommended a ban on the routine addition to animal feeds of antibiotics used to treat human and animal diseases. The British government swiftly adopted the recommendation, and other European governments have since followed suit.

Following publication of the Swann Report, FDA established a task force of its own to look into the matter, and in February 1972, the task force recommended that restrictions similar to those adopted in Britain should be imposed in the United States. But the task force put an important proviso on that recommendation: the ban should be delayed for up to two years to allow the drug industry time to try to prove its claim that the use of antibiotics in animal feeds does not lead to human health hazards. The deadline slipped by, but FDA took no formal action and non-therapeutic drug use on the farm has continued to increase. According to FDA estimates, 1.2 million pounds of antibiotics were added to animal feeds in 1960, 7.3 million pounds in 1970, and an average of 7.7 million pounds each year between 1971–75.

The first public indication that FDA hadn't completely forgotten about the problem came last April when Donald Kennedy, the new FDA commissioner, announced that FDA was finally prepared to act. "Although we can point to no specific instance in which human disease is more difficult to treat because drug resistance has arisen from an animal source, it is likely that such problems have gone unnoticed", Kennedy said. "The benefit of using these drugs routinely as over the counter products to help animals grow faster or in prophylactic programs does not outweigh the potential risk posed to people", he added. Consequently, Kennedy announced that FDA would seek to eliminate use of penicillin and severely restrict use of tetracyclines in animal feeds. (It should be noted that some antibiotics most critically needed for therapeutic use in man and animals, such as chloramphenicol, semi-synthetic penicillin, gentamycin, and kanamycin, have never been allowed in animal feeds.)

Last week FDA formally published a regulation barring use of penicillin in animal feeds, (it is used chiefly for pigs and poultry), and a similar ban

Sorry, for copyright reasons some images on this page may not be available online

Popperfoto

Donald Kennedy: new commissioner

on tetracyclines is expected in the next few weeks. Other antibiotics are under consideration, but FDA officials say they do not expect to take action on them in the near future.

The FDA decision is sure to be controversial, and the agriculture and pharmaceutical industries are expected to challenge it in court. One reason is the economic impact. FDA reckons that eliminating penicillin alone will cost some \$12 million in lost sales and increase feed costs: the impact of eliminating tetracyclines is expected to be even higher.

The industry is expected to argue on several different grounds. First, there is no demonstrated case in which antibiotic resistance derived from lacing animal feeds has led to untreatable bacterial infection in humans. Second, that resistance to penicillin and tetracyclines is already so widespread that banning their use in animal feeds will make little difference. And third, the chief reason why antibiotic resistance has become such a serious medical problem is that antibiotics have been overused and inappropriately prescribed by the medical profession.

The industry has a right to appeal the FDA decision by filing a complaint and requesting a public hearing, a process which could take up to a year and use of the drugs in animal feeds would be permitted until the appeal is exhausted. After that, the industry could go to court in an attempt to overturn the ban.

Even if the FDA action is ultimately upheld, the use of antibiotics in animal feeds will not be ruled out entirely. The pharmaceutical industry has produced a number of antibiotics which are not used to treat human disease and which may therefore be safer replacements for penicillin and tetracyclines. According to FDA officials, those antibiotics are not in widespread use because they are more expensive than penicillin and tetracyclines. □

USSR

Discrediting the dissidents

Vera Rich reports on efforts within the Soviet Union to play down the strength of dissident opinion

THIS autumn, the Soviet Union will be celebrating the sixtieth anniversary of the October Revolution. Unlike previous anniversaries, this one will take place against a background of dissident opinion which, to the obvious embarrassment of the official concept of Marxist-Leninism as a 'scientific' basis for society, includes among the dissidents a number of scientists.

Accordingly, it would seem that there is a last-minute campaign to reduce the impact of the dissident movement on the outside world before the celebrations commence, by means of media propaganda.

A recent article in the prestigious *Literaturnaya Gazeta* attacked the Oxford conference on physical chemistry and hydrodynamics which was held in honour of the sixtieth birthday of Corresponding Academician Veniamin G. Levich. Totally ignoring the scientific work of the meeting (for which see *Nature* 268, 298-299, 28 July, 1977), the article set out to prove that the organisers, Sir Derek Barton and Professor Brian Spalding, had been little more than figure-heads for organisers

"far removed from science". It cited the fact that a few of the Soviet scientists invited were already dead. Professor Spalding said that he had found the names of the Soviet scientists in a 1976 edition of the *World of Learning* and added that a few clerical errors were inevitable in organising a large conference.

Commenting on the article's other main charge, that Professor Levich had long ceased to work in science, Professor Spalding pointed out that this was a particularly wicked accusation, since it was the Soviet authorities' harassment that hindered Levich from continuing his scientific work. Spalding further noted that the author of the article seems to have violated the secret archives of the Academy of Sciences, concerning Levich's failure to be elected full Academician. Since the Academy elections are still by secret ballot, this seems ominous.

Somewhat similar in tone were the numerous broadcasts (largely in western languages) which preceded the Honolulu Congress of the World Psychiatric Association (WPA). The message was simple: Soviet psychiatry is a purely therapeutic activity, and all suggestions of the psychiatric repression of dissidents are simply propaganda. A number of western psychiatrists were cited

as having concurred in the diagnoses given to alleged dissidents. However, Dr Avtandil Papiashviliar, a psychiatrist who recently emigrated to the West, said in London that such claims were "a straightforward lie". He further maintained that during the WPA Symposium in Tbilisi, in 1974, the KGB had arranged for a genuine mental patient to break into the meeting, claiming to be persecuted by the police, in the hope that the visiting delegates would first protest, and then have to admit that the man was indeed in need of psychiatric attention. In the event, the Honolulu Congress passed (albeit narrowly) a motion censuring Soviet psychiatric repression of dissidents and the Soviet delegation decided not to secede from the WPA.

Honolulu can rightly be claimed as a success for all those concerned with the human rights movement in the Soviet Union. No such success, however, can yet be claimed for the recent Anatolii Shcharanskii hearing in the Swedish House of Parliament. Shcharanskii, a young mathematician who was a founder member of the Moscow 'Helsinki Monitoring Group', was arrested last March accused of spying for the CIA. The Stockholm hearing, before Hårje Stenberg, Master of the Swedish Appeal Court, not only presented circumstantial evidence of Shcharanskii's innocence, but also pressed for his human right of a fair trial. □

NEW ZEALAND

●The latest budget from the New Zealand government gives a substantial fillip to the country's Department of Scientific and Industrial Research. Although the country is going through difficult economic times expenditure on science-related activity is to rise by 16% to \$NZ 35 million for the financial year 1977-78 (£1=\$NZ 1.78). That increase should keep pace with inflation and provide for some real increase as well but staff figures in the Department continue to be held at just over 2,000.

The budget allocation breaks down into \$NZ 14 million on agricultural work; \$NZ 3.15 million on energy; \$NZ 3.41 million on manufacturing; \$NZ 6.81 million on the natural environment; and the balance on miscellaneous activities. The department is making a special effort on energy research in efforts to discover long-term alternatives to New Zealand's reliance on overseas sources of fuel.

Meanwhile university research personnel gained some benefit from the budget, having gone through a very

lean time. Last year the Universities Research Fund was denied funds by the government in a savage attack on spending. This budget allocates the Fund \$NZ 750,000.

●A master file of over 14,000 earthquakes in earthquake-prone New Zealand has been established. The file has been placed on computer tape and runs from the first recorded earthquake in AD 1460 to the most recent shocks of 1975. The file will greatly assist the task of scientists working out the geographic patterns of earthquakes and researchers in the construction industry.

●A Royal Commission on Nuclear Power is sitting in New Zealand in an effort to discover the real needs of the country before any step is taken to set up nuclear power stations. The Labour opposition has been vigorously opposed to nuclear power but the Conservative government is waiting on the Commission. The Minister of Energy Resources, Mr George

Gair, says he believes the country has at least five years in hand before any decision need be taken.

●The government has set up a working party to examine the need for legislation to control genetic engineering experiments which are going on in New Zealand. So far, the few scientists working in the field have agreed to abide by the Williams Committee guidelines from the United Kingdom, but now the government believes further study is needed.

●Just to produce and supply electricity to New Zealand's three million people is calculated to cost about \$100 for every person. In an effort to recoup the costs the government continues to allow energy prices to rise. But in the search for alternative sources the government will control a major on-shore drilling programme, take over an off-shore mining company and the Natural Gas Corporation which controls the Maui gas field.

Bruce Wallace

IN BRIEF

Instant data

By 1979 experimental data should be travelling between four major European high energy physics laboratories almost as fast as it can be processed. Under a project called STELLA (Satellite Transmission Experiment Linking Laboratories), which will involve the UK's Rutherford Laboratory, DESY in Germany, Saclay in France and CERN in Geneva, the data will be transmitted via the Orbital Testing Satellite, the European Space Agency's communications satellite scheduled for launch on 8 September.

The project has recently won approval from the Council of Ministers of the European Economic Community for funding worth SF 1.2 million, which will be spent on building a small satellite earth station and associated computer at CERN. It should demonstrate to industry and Europe's posts and telecommunications organisations, the value of using the new design of

European satellite for transmitting data almost instantaneously at very low error rates.

Spacelab selection

Last week, 12 European countries put forward 53 candidates for selection as Europe's first astronaut. Whoever is chosen will travel with one American on the first mission of the European Space Agency's (ESA) Spacelab, a reusable space laboratory which will be put into earth orbit in 1980 by NASA's Space Shuttle.

By the end of this year the 53 candidates will have been whittled down to six who will undergo further tests by NASA and ESA. Before the middle of next year, three will have been chosen for intensive training for the first Spacelab mission. A few months before the launch, one will be finally selected to travel in Spacelab while the other two work on ground-based activities.

The UK has put forward five candi-

dates, the maximum number any member country of ESA was allowed to submit. They are William Grut from Surrey University, Geoffrey Firmin from Associated Nuclear Services Ltd, Arthur Ince from East Birmingham Hospital, Keith Mason from the Mullard Space Science Laboratory and M. J. Rycroft from Southampton University.

Whilst Europe has been selecting candidates for Spacelab, NASA has been working out how much to charge customers for flights on the Space Shuttle. The cheapest fare, at \$10,000, is likely to be for payloads involving research, weighing less than 200 pounds and with a volume less than five cubic feet. The maximum fare will be around \$21 million for full use of the Shuttle by non-US government customers. NASA already has 40 payloads for 1980, the first year of the Shuttle's operation. Spacelab is due to go up on Space Shuttle flight 11 for a fare somewhere in the middle of the price range.

AGRICULTURAL productivity throughout the world is increasingly dependent on the liberal use of chemical fertilisers, particularly those containing nitrogen. This is true of the sophisticated farming systems in Europe and North America, and where the 'green revolution' is successful in developing countries. A substantial amount of energy and oil goes to produce these fertilisers, and therefore many people are doubtful whether their continued and increasing use will be possible in an energy-hungry world in the twenty first century.

As I showed on 25 August, oil-hungry tractors actually use less energy than horses on the farm. Chemical fertilisers may, on balance, increase the amount of energy available to the farmer and to the population he serves. Returning to our Kalahari bushman, so idolised by some ecologists for his allegedly-economical use of energy; he certainly extracts many more calories than he puts into the system, but he only obtains enough food from a square mile or more of territory to feed himself. The modern arable farmer feeds several people on the produce of a single acre. He puts more into the system, but he takes a great deal more out. The difference between his energy expenditure, in cultivation, spraying and harvesting, and that stored in his crops, is several hundred times that of the bushman's total productivity per unit area.

In the UK we use less than a million

tons of oil to produce over a million tons of nitrogenous fertiliser. Only a part of this is used on cereal crops which now yield annually some fifteen million tons of grain and ten

Food for energy



KENNETH MELLANBY

or more million tons of straw. Without the fertiliser the yields would be reduced by a factor of two or even three. The energy wasted by burning some three million tons of unwanted wheat straw could, in theory, power all our tractors and the factories making our manures.

Unfortunately we cannot, at present, use the straw in this way, but we should surely try. To turn straw, readily available on the surface of the

ground, into useable fuel should be a simple problem for our research workers to solve. A fraction of the money used for more esoteric agricultural research would be well invested to this end.

Although arable farming uses energy efficiently, further improvement is desirable, if only to reduce costs. In Britain we waste a substantial amount of organic manure which would save synthetic chemicals and might improve soil structure, but which, when fuels are cheap, is expensive to use. As fuel prices rise, this waste will be avoided. Cultivation may be further reduced when weeds are controlled by modern herbicides. Though it will probably be a long time before research to make it possible for cereals to fix atmospheric nitrogen gives positive results, we now make too little use of the ability of leguminous crops to perform this function. World phosphate supplies are limited, but fungal mycorrhizae may be increasingly used to mobilise insoluble compounds in the soil. All the indications are that in future agriculture will use less and not more energy, without any loss in productivity.

Thus though we may face a global energy shortage in the next twenty five or thirty years, so that the extravagant use of cars and aeroplanes may have to be restricted, and we may have to stop overheating our houses and offices, we should, with a rational system of priorities, have plenty of energy to produce our food.

correspondence

Alaskan earthquake

SIR,—The item on Alaskan earthquakes by P. J. Smith (7 July, page 12) is misleading—the point at issue is not the frequency of seismic events in Northern Alaska, but their magnitude.

The section of the pipeline of which he writes was required to be designed to withstand an earthquake of magnitude 5.5 without spillage; the federal requirements were only drawn up after extensive analysis by a variety of groups not depending solely on seismic data. Obviously these people appreciated that the absence of recorded activity was more apparent than real. I can see no reason to assert, as Smith does, that a report on the frequency of seismic events in the magnitude range 1 to 4 for a section designed to resist magnitude 5.5 shocks reveals an example of oversimplification through ignorance.

I might add that many crude oil pipelines around the world were built before good seismic data became available (for instance in Iran early in the century) and there is no record of any of them ever having ruptured as a result of an earthquake.

Yours faithfully,

F. G. LARMINIE

British Petroleum, London, UK

China and scientific unions

SIR,—In your editorial (28 July, page 283), you accuse The People's Republic of China of "trying to import" politics into the international scientific unions. You ignore the fact that politics must inevitably play a role in the unions as they are at present constituted. For example, how can a union, composed of member countries and financed by their governments, fail to consider the question of what is meant by a country?

In discussing Chinese representation in the unions one should remember that Taiwan has never been claimed to be other than a province of China by the authorities either in Peking or Taipei. Thus recognising Taiwan as an independent country would place the international unions in the strange position of giving Taiwan a status not claimed for it by any international authority including the United Nations and UNESCO, or even by its own administration. Although this is a course of action that was taken by

several of the unions (eg the International Astronomical Union in 1958), such a decision withdrew the right of the Academia Sinica in Nanking to represent a part of China which it had previously represented and in my view thereby often violated the statutes of the union.

Such actions took place during the fifties when a successful campaign was instigated by the US State Department to secure the membership of China in the unions for representatives of the Academia Sinica in Taipei. Often the Taiwanese applied and were admitted to membership of unions for which any relevant activity in Taiwan was miniscule or non-existent. The acquiescence of ICSU and its unions in this attempt to isolate the Chinese from the international scientific community is responsible for the bitterness with which many Chinese scientists still view ICSU and the insistence with which they demand that the previous decisions be reversed.

Yours faithfully,

GEORGE K. MILEY

Leiden, Netherlands

No arms link

SIR,—In your 'in brief' column (18 August, page 582) you state that "African National Congress officials are reported as saying that 15 German research institutions, including the Max Planck Institute for Nuclear Physics at Heidelberg, have contributed to South Africa's arms programme". We would like to state that the Max Planck Institute for Nuclear Physics is devoted only to basic research in the physics of nuclear structure and nuclear reactions. This research is completely unrelated to arms production.

Yours faithfully,

U. SCHMIDT-ROHR

*Max Planck Institute for
Nuclear Physics,
Heidelberg, West Germany*

Sunflower: a misnomer?

SIR,—It is commonly believed that the movement of the sunflower (*Helianthus annuus*) is a typically heliotropic movement and that with a certain lag, heads follow the path of the sun across the skies in the wake of its daily east-west movement. Observations on a field of

sunflowers growing in the essentially cloudless Mediterranean summer climate at the Ma'abaroth kibbutz settlement in the Sharon plain in Israel reveal that during the night the extent of sunflower head movement is more rapid than during the day.

At dusk all heads do indeed face west, but by 2 am, before any trace of light on the horizon, all heads had reverted to an east facing position. Calculated average velocity of movement during daylight is $\pm 13^\circ$ per h as against $\pm 26^\circ$ per h in the dark.

Furthermore, when complete rotation towards the east had occurred, by 2 am the heads were held at $\sim 40^\circ$ – 45° above the horizontal and upon the appearance of the sun heads 'nodded' eastwards to the extent of a further 15° , as if slightly bowing to greet the rising sun, and only subsequently started their daily journey westwards.

These observations suggest an endogenous 'spring' mechanism, apparently wound up by the sun and released in the dark or light period. The fact that the night movement is twice as rapid as that of the day seems to support the concept that the 'spring' release occurs in the dark and the winding in the light since unhindered mechanical spring release is presumably more rapid than winding, which demands active energy input. If, however, in the future the converse proves to be true, botanists may possibly reach the conclusion that the designation 'sunflower' (of similar etymology in most languages) may be a misnomer and in its stead 'nightflower' may be suggested.

Yours faithfully,

YA'ACOV Y. LESHEM

*Bar-Ilan University,
Ramat-Gan, Israel*

'The Iron Sun'

SIR,—Roman Znajek, in his review of my book *The Iron Sun* (30 June, page 867) suggested, no doubt unintentionally, that I had misquoted Einstein. In fact, I had made a proof-reading error. The passage in Einstein's *The Meaning of Relativity* to which I had wanted to draw attention is on page 26 of the 1973 Chapman and Hall Science Paperback edition.

Yours faithfully,

ADRIAN BERRY

London W8

news and views

Predators that switch

from Robert M. May

PREDATION is an important factor in enhancing the diversity of plant and animal communities. One classic example of the way predation can promote coexistence among competitors that would otherwise not persist together is Paine's (*Am. Nat.* **100**, 65; 1966) study of what happened when the dominant predator (a starfish, *Pisaster*) was systematically removed from an intertidal community of marine invertebrates: in less than 2 years, the community collapsed from 15 to 8 species. A variety of other broadly similar examples, from communities on the land and in the sea, have been drawn together by Connell (in *Ecology and Evolution of Communities*, (eds Cody & Diamond) Harvard University Press, 1975).

Suppose we have two or more competing species, one of which would exclude the other in the absence of predation. One pervasive mechanism whereby a predator can enable these competing prey species to coexist is by directing a disproportionately large amount of its attention to the prey species that happens to be most abundant at any one time. This 'switching', to concentrate attacks largely on the most common prey species, may be a simple consequence of the predator's searching behaviour; for example, many predators form a 'search image', which is likely to be set by the locally most abundant prey species. Such patterns of 'switching predation' are common among vertebrate predators (Holling *Can. Ent.* **91**, 293; 1959; Murdoch *Ecol. Monogr.* **39**, 335; 1969), and also among many invertebrate ones (Murdoch & Oaten *Adv. ecol. Res.* **9**, 1; 1975; Hassell Lawton & Beddington *J. Anim. Ecol.* **46**, 249; 1977).

There is no doubt that predation with switching exerts a generally stabilising effect on the dynamics of a community. Elementary accounts of the phenomenon are, however, liable

to two common misapprehensions, which several recent papers have helped to clarify.

The first misunderstanding concerns the extent to which switching predators contribute to the overall stability within the single trophic level of the competing prey species. Too often, simple accounts portray switching as a kind of Universal Stabiliser. In fact, as first emphasised by Steele (*The Structure of Marine Ecosystems*, Harvard University Press, 1974), although switching in a 1-predator/ n -prey system (in which one species of predator preys on n species of prey) will tend to stabilise the competitive interactions, it can result in overall instability if each individual 1-predator/1-prey system is by itself unstable. Steele lucidly combines qualitative reasoning, analytic results and a computer simulation to show that in this case the internal dynamics within the prey trophic level will settle to some stable state (some stable proportion of each prey species), but nonetheless the overall prey/predator system remains subject to the basic instability manifested by the individual prey/predator interactions. Steele's discussion is for overlapping generations, with differential equations; another illuminating study is by Comins and Hassell (*J. theor. Biol.* **62**, 93; 1977), who consider a 1-predator/2-prey system with non-overlapping generations obeying difference equations. In the model discussed by these authors, it can be seen explicitly that predator switching enlarges the domain of parameter space wherein the two prey species can coexist, yet it has no influence on the boundaries of the domain of parameter space wherein the overall prey/predator system is stable. These themes are succinctly summarised by Murdoch (*Am. Nat.* **111**, 383; 1977).

The second misunderstanding concerns the extent to which generalised predation, without any switching, can help competing species to coexist. In very simple mathematical models

(where all prey have the same intrinsic growth rate) a perfectly generalised predator, which attacks all prey species in impartial fashion, makes no difference to competitive coexistence among the prey; the internal dynamics within the trophic level of the prey is unaffected by predation of this evenhanded kind (Van Valen *J. theor. Biol.* **44**, 19; 1974; May in *Some Mathematical Problems in Biology* **6** (ed. Cowan) Amer. Math. Soc., Providence, Rhode Island, 1974). But a small amount of switching behaviour on the part of the predator alters this situation, and can make possible a significantly greater degree of 'niche overlap' among the competing prey species than would otherwise be possible (Roughgarden & Feldman *Ecology* **56**, 489, 1976; Janson, in manuscript, 1975; Murdoch and Oaten, *op. cit.*). Roughgarden and Feldman's study is a particularly clear and rigorous account of the way predator switching can permit increased niche overlap among the prey; yet at least one recent publication quotes it as affirming the broader and unqualified conclusion that "predation increases niche overlap."

Of course, predation can modify the basic conditions under which competition takes place, even if there is no switching. Thus generalised predation may promote coexistence if, among the prey, the less able competitors have higher intrinsic growth rates. Specific examples arise in pastures subject to 'predation' by grazing. Here species diversity can be increased because plants that would be shaded out, or otherwise outcompeted, are given a chance. Harper's (in *Diversity and Stability in Ecological Systems*, US Natn. Bur. Standards, Springfield, Virginia, 1969) review of such situations includes deliberate and accidental (following myxomatosis) experiments where rabbits were withdrawn, and controlled experiments in pastures grazed by sheep. Paine's example is of this general kind.

The above discussion (despite its

pedantic nit-picking) is directed mainly at affirming that predation in general, and particularly predation with switching, can make it easier for prey species to coexist, and thus lead to communities that are more diverse at the species level. If we shift our perspective to focus on the coexistence of different genotypes within a single prey species, analogous considerations arise. By concentrating its attacks on the most common genotype, a switching predator can enable the rarer genotype to enjoy higher fitness; this amounts to 'frequency-dependent natural selection', and is a powerful mechanism whereby genetic polymorphism can be stably maintained. This case has been argued by Clarke (*Sci. Am.* **233** (2), 50; 1975), who emphasises that predator switching is only one of a variety of biologically plausible ways in which frequency-dependent natural selection may be realised. □

Two-faced neurones

from Key Dismukes

THE classic concept of neuronal operation—dendritic summation of synaptic inputs, generation of all-or-none action potentials, propagation along axons, and release of neurotransmitter from terminal boutons—increasingly seems to be an oversimplification. For several years evidence has been accumulating for an array of information processing operations that go beyond the framework of traditional neuronal doctrine (Schmitt, Dev & Smith *Science* **193**, 114; 1976). In the retina, for example, the dendritic projections of axonless local neurones interlace with the synaptic connections of other cells communicating bidirectionally without the use of action potentials.

Recent biochemical studies suggest that the traditional view of the neurone as transmitting information in only one direction will have to be modified. The dopaminergic cells of the substantia nigra have provided the most striking evidence, recently reviewed by L. Iversen (at the Neurosciences Research Program Study Program at Boulder, Colorado in July (to appear in *The Neurosciences: Fourth Study Program* (eds Schmitt & Worden) MIT Press), in whose laboratory much of the work has been done.

Cytochemical studies in several laboratories have indicated the presence of dopamine and the enzymes of its bio-

synthetic pathway not only in cell bodies of the substantia nigra, but also in dendritic fields. This surprising discovery raised the suspicion that dopamine might be released from dendrites, in addition to its normal secretion from axon terminals. To investigate this possibility, Iversen's group studied the release of dopamine from nigral tissue *in vitro* by applying the superfusion chamber techniques that have been widely used to elucidate mechanisms of neurotransmitter secretion. In contrast to the usual conditions, the tissue in these experiments contained no axon terminals bearing the transmitter being studied (the dopaminergic axons of the substantia nigra terminate remotely, in the forebrain). Thus, if dopamine release occurred, it would have to be from the dendrites and/or the cell bodies.

Dopamine was in fact found to be released from the substantia nigra, in a fashion closely paralleling that seen in the neostriatum, which does contain dopaminergic nerve terminals (Geffen *et al. Nature* **260**, 258; 1976). Depolarising tissue fragments by raising the external concentration of K^+ produced a substantial efflux of dopamine. This efflux was blocked by removing Ca^{2+} from the superfusion medium, or by Mg^{2+} concentration. Ca^{2+} flow is known to be a central mechanism in neurotransmitter release; thus dopamine efflux from substantia nigra satisfies one of the major criteria for distinguishing the specific secretion of a transmitter molecule from nonspecific leakage through membranes.

Most of these experiments were performed by labelling endogenous dopamine pools with 3H -dopamine and following the appearance of radioactivity released into the superfusion fluid. This was possible because the substantia nigra possesses a high-affinity uptake mechanism for dopamine. This is an interesting fact in itself, because these specialised uptake systems are generally associated with nerve terminals, and are thought to provide a means of inactivating transmitter molecules released into the synaptic cleft. Verification of the results obtained with 3H -dopamine *in vitro* was obtained by using a highly sensitive radioenzymatic assay to demonstrate the minute efflux of endogenous dopamine released by depolarisation. Release of dopamine from substantia nigra has recently been directly demonstrated *in vivo*. Nieoullon *et al.* used push-pull canuli to superfuse the substantia nigra with 3H -tyrosine and to monitor the efflux of 3H -dopamine synthesised intracellularly (*Nature* **266**, 375; 1977). As in *in vitro* experiments, elevated K^+ concentration stimulated the release of 3H -dopamine.

Does dopamine released from the substantia nigra come from dendrites or from cell bodies? That question was answered by making use of the segregated topography of this nucleus. Dopaminergic cell bodies are confined to a central *zona compacta*, whereas dendritic processes invade both that zone and the surrounding *zona reticulata*. 3H -dopamine was taken up and released by depolarisation in both regions indicating that dendrites are indeed capable of secreting neurotransmitter.

What are the targets of dopamine released from the substantia nigra? Evidence suggests that one possibility is the GABA-containing axons from the striatum which terminate in the substantia nigra. Dopamine was recently reported to stimulate the overflow of 3H -GABA from nigral tissue *in vitro* (Reubi, Iversen & Jessell *Nature* **268**, 654; 1977). This stimulation was blocked by antagonists of dopamine, or by lowering Ca^{2+} concentration, indicating that the effect was specific.

Iversen also reported that modulation of GABA release from striato-nigral terminals is also suggested by lesion studies. Neural responses to dopamine seem generally to be mediated by cyclic AMP, whose formation is controlled by adenylate cyclase linked to dopamine receptors. Lesions of striato-nigral pathways containing GABA were found to cause disappearance of dopamine-sensitive adenylate cyclase in the substantia nigra. In contrast, destruction of substance P pathways or of dopaminergic cells themselves did not reduce activity of this adenylate cyclase.

Aghajanian and Bunney have shown that microiontophoretic application of dopamine to the substantia nigra *in vivo* inhibits the firing of dopaminergic cells (*Neuyn-Schmeidebergs Archs Pharmac.* **297**, 1; 1977). This finding, coupled with the GABA studies, suggests a functional role for dendritic release of dopamine. GABA should inhibit the firing of dopaminergic cells in the substantia nigra. Dendritic release of dopamine, by stimulating GABA release from pre-synaptic terminals, could provide a local feedback mechanism to modulate the activity of dopaminergic neurones.

Morphological features suggest that dopamine may be released from dendrites by a different mechanism from that in axon terminals. Iversen reported that electron micrograph studies give no suggestion that dopamine-bearing dendrites contain synaptic vesicles. (This raises a puzzling question about the function of Ca^{2+} , whose role in secretion is believed to involve triggering extrusion of vesicle-bound

transmitter). Nor is there any indication that dopaminergic dendritic elements form synaptic connections with other cells. However, several authors have suggested, on the basis of morphological evidence, that amine neurotransmitters in the brain can be released nonsynaptically (Chan-Palay *Cerebellar Dentate Nucleus* 418-425, Springer Verlag, Berlin, 1977, and references therein). This mode of secretion could be aimed at more distributed targets, and might represent a form of communication intermediate between the exclusive addressing of synapses and the broadcasting of hormones.

Giant molecular clouds

from M. G. Edmunds

A workshop on Giant Molecular Clouds in the Galaxy was held at Gregynog Hall, Newtown, Wales from 8-13 August 1977. It was organised by University College, Cardiff.

THE recent rapid growth in knowledge of the distribution of the carbon monoxide (CO) molecule in the Galaxy by observation of its line radiation at millimetre wavelengths has led to the discovery of a new and very significant component of galactic structure; the Giant Molecular Cloud. The importance of CO is as an indicator of the otherwise virtually unobservable hydrogen molecule H_2 . Where CO forms, it is expected that H_2 will also form, and since hydrogen is by far the most abundant element in the Galaxy, the CO maps can trace the overall density of the interstellar medium. The result of surveys such as those outlined by P. M. Solomon (Stonybrook, New York) suggest that there exist very large 10-80 pc clouds of molecules with masses which are typically 10^5 solar masses and may even reach as high as 10^7 solar masses. Of particular importance have been new maps in ^{13}CO radiation which do not suffer from the severe optical depth uncertainties of ^{12}CO but confirm the large masses of the clouds. The densities are high in these clouds, on average about 700 molecules per cubic centimetre, compared with a value of perhaps one atom per cubic centimetre in the general interstellar space between the clouds.

M. G. Edmunds is in the Department of Applied Mathematics and Astronomy at University College, Cardiff.

Infectious provirus synthesised *in vitro*

from Robin Weiss

ON page 122 of this issue of *Nature* Ellen Rothenberg and her colleagues describe the *in vitro* synthesis of infectious DNA of murine leukaemia virus. Although this feat of high fidelity reverse transcription was first announced more than a year ago, and this paper comes as no surprise, it does mark the culmination of several years investigation of reverse transcriptase activity *in vitro*. The successful transfection of a retrovirus by DNA extracted from infected cells was first achieved by Hill and Hillova (*Nature new Biol.* **237**, 35; 1972) and it constituted a formal proof of Temin's DNA provirus hypothesis. Now the MIT laboratory's improved techniques have yielded complete DNA transcripts *in vitro* which show single-hit dose response for infectivity.

The infectivity of the transcripts is resistant to protease and ribonuclease but is completely destroyed by deoxyribonuclease. The structure of the infectious DNA molecule is curious. It appears that a full-length negative strand is synthesised and shorter segments of positive-strand DNA are formed in duplex with it. Transcripts synthesised in the presence of actinomycin D are not infectious; whether this is attributable to the absence of positive-strand DNA or to incompleteness of the negative-strand transcripts is not clear. The data and their significance are so clearly presented that any further commentary here is superfluous.

The importance of the clouds lies not only in their being easily the most massive individual objects in the galactic disk, but also because they are regions of very active star formation.

The actual number of these clouds in the Galaxy was a matter of some contention, depending to some extent on whether the giant clouds are regarded as a single gravitationally bound system, with complex subcondensations, or as a superposition of smaller independent subunits as proposed by M. Gordon (US National Radio Astronomy Observatory). Most participants were content to accept that each cloud really is a single large complex, and the probable number would then be of order 1,000-4,000, constituting the major part of the mass of interstellar material in the Galaxy. The clouds are not uniformly distributed, showing a very marked 'ring' structure with a concentration of the clouds between 4 to 8 kpc from the centre. It is inter-

esting that the distribution of clouds has not yet been shown convincingly to indicate spiral structure in the disk, although A. Pedlar (Jodrell Bank) claimed some slight evidence from a formaldehyde molecule survey. The Galactic Centre itself is the other major concentration of molecular clouds, a fascinating complex reviewed by N. Scoville (University of Massachusetts) containing as much as 10^7 to 10^8 solar masses, with recent data indicating that a model with an expanding spiral pattern extending right in to the centre may be more attractive than previously proposed expanding disk models.

The formation of stars in the molecular clouds is clearly demonstrated by the existence of infrared sources, caused by the inevitable heating of gas and dust as it collapses under its mutual gravitational interaction, and further heating once the stars have started nuclear energy generation. One obvious site for star formation in the clouds is the characteristic small 'cores' where higher than average temperatures and densities indicate that collapse may be taking place. Much evidence was presented that although low mass stars may form throughout the clouds, the high mass stars over about 12 solar masses form only near the edge of the clouds. B. Elmegreen (Harvard University) and C. Lada (Center for Astrophysics, Cambridge, Massachusetts) claimed this as good evidence for a mechanism of inducing the collapse of further protostars at the edges of the clouds by the effect of compression between shock and ionisation fronts driven into the clouds by radiation, once one bright massive star has formed. The existence of ionised hydrogen regions at the edge of molecular clouds seems very common, and F. Israel (Caltech) presented evidence that most bright, dense regions are of this type, with ionised radiation eating its way into the cloud.

But the mechanism which actually drives the typical subcondensation of a cloud into a state where it will collapse into a star, or cluster of stars, was the subject of much discussion. The effects of the shock wave from a supernova explosion were championed by G. Assousa (Carnegie Institution, Washington), and shocks induced by stellar winds from massive stars maintained as a possibility by L. Blitz (Columbia University) when presenting CO maps of many local molecular clouds. P. Woodward (Leiden) described his extensive numerical calculations of the collapse of clouds induced by the passage of the large-scale density-wave pattern which is believed to maintain a spiral structure in the Galaxy. He received some support from

Elmegreen's observation of what looks like a temporal star formation sequence along the Galactic plane from an infrared source at one end of a giant molecular cloud to a young ionised hydrogen region M17 at the other end of the cloud. But the star formation in many other clouds, such as Orion, shows no particular preference for the direction along the Galactic plane.

Probably all of the mechanisms, and simple cooling or collisions, are important in instigating the collapse of the clouds. Once collapsing it is possible that the collapse phase may be fairly insensitive to the inducing mechanism. Attempts to follow the subsequent dynamical and chemical evolution described by A. Glassgold (University of Oxford) and others are idealised and have not yet reached the stage where they can be reliably compared with observation. The effects of magnetic fields are beginning to be incorporated, but the particular physical and chemical conditions peculiar to the molecular clouds must also be incorporated.

Very small scale structure in the star-forming areas of the clouds can be obtained by observations of radio lines of molecules, such as H_2O , SiO and OH , formed under maser conditions. D. Downes (Bonn) showed some beautiful very-long-baseline-interferometry H_2O maser maps of several molecular clouds compiled by American-German-Russian-Swedish collaboration. The maps show detail down to milliarc s, corresponding to sub-Solar System sizes in molecular clouds. Downes proposed that the best interpretation of the motion of the clusters of masering regions is as the effect of the shell containing the masers expanding and breaking up after the formation of a star at the centre. Scoville, in looking at the high velocity gas observed in ^{13}CO mapping of the Orion molecular cloud, proposed on energetic grounds that an explosion must have occurred (perhaps a supernova) since radiation pressure from a massive star would be insufficient to drive the matter outwards without the infrared luminosity of the source exceeding its observed value.

Complex molecules may be of great relevance for the composition of interstellar dust. F. Hoyle and N. C. Wickramasinghe (University College, Cardiff) proposed polysaccharides, possibly formed by surface polymerisation of the common formaldehyde molecules, as excellent candidates to explain the 10, 3 and $3.4\ \mu\text{m}$ features observed in the infrared spectra of compact sources. They also suggested that organic ring structures may explain the $2,200\ \text{\AA}$ ultraviolet feature in the interstellar extinction curve. Support for the idea that a fairly volatile component

does indeed produce the $2,200\ \text{\AA}$ feature was provided by Israel, who described a preliminary analysis of ultraviolet satellite observations of the galaxy M101 which suggests that the feature vanishes near young hot stars, an effect already noticed in the 30 Doradus nebula in the Large Magellanic Cloud.

Not all star-forming molecular clouds in the Galaxy are massive. Elmegreen described a complex in NGC 7023 of about 1,000 solar masses, and M. Walmsley (Bonn) a molecular cloud in Taurus of only about 1 solar mass. In both these clouds, low mass star formation seems to be occurring.

Many questions were necessarily left unanswered. How do the massive clouds form? Why are they such long-lived structures, with their estimated lifetimes greatly exceeding the expected gravitational collapse time, yet allowing some star formation to proceed? Giant Molecular Clouds have established themselves as a primary component of the Galaxy, and their study will provide many clues in understanding the mechanism of star formation. □

Actin cables

from Roger Craig

THE view that muscles contract by a sliding of the actin filaments past the myosin filaments is firmly entrenched in our minds. So beautifully does it explain both structural and biochemical observations, that the possibility that actin or myosin in non-muscle cells might perform in any different way has, until recently, hardly been countenanced. But actin, for one, does now seem to function in some cells in a distinctly non-muscular fashion.

In muscle, actin occurs as filaments—helices of globular actin monomers—which function in two intimately related ways: as structural components able to bear tension, and as activators of the energy-releasing ATPase cycle of myosin. In non-muscle cells actin can occur in different forms depending on the immediate and local needs of the cell. One of the most important of these seems to be cables of parallel filaments, which are found in intestinal microvilli, in tissue culture cells, in the acrosomal process of some invertebrate sperm, in the cytoplasm of plant cells, and in many other situations.

Although myosin occurs in association with actin in many non-muscle cells (although generally in minute

amounts), there are some cells rich in actin where myosin has not been detected. The question therefore arises: does actin perform a non-motile role in some cells, or does it perform a motile function without the participation of myosin? Actin cables should provide some answers to these questions: since they are a different structural state of actin from that found in muscle, it would not be surprising if they also differed functionally.

What are the structural advantages of a tightly packed cable of actin filaments? The most obvious is one of rigidity: filaments bound together laterally will form a much more rigid structure than an array of unconnected filaments. Such a structure should be able to support a compressive load in addition to the tensile forces actin can sustain in muscle contraction. The finding of a criss-cross network of such cables in many cells has led several authors to suggest that actin in some instances may fulfil a purely structural role, as part of a cytoskeleton which maintains cell shape and strength (see Clarke & Spudis *A. Rev. Biochem.* **46**, 797; 1977). An instance where actin seems to have a motile ability not dependent on myosin is the acrosomal reaction of some invertebrate sperm (Tilney in *Molecules and Cell Movement* (eds Inoué & Stephens), Raven Press, New York, 1975). The acrosomal process, which penetrates the outer layers of an egg during fertilisation, forms rapidly, growing up to $90\ \mu\text{m}$ in only 10 s; and it consists essentially of a beautifully ordered cable of actin filaments. Two mechanisms for the acrosomal reaction have been demonstrated. In echinoderm sperm the process develops by the organisation of actin into the filamentous form from an amorphous state. In the horseshoe crab *Limulus*, the cable is preformed but coiled in the head of the sperm. The process is extended by a rearrangement of the filaments in the cable, which emerges from the tip of the sperm.

Cables of filaments are clearly an important form of actin in non-muscle cells. A recent paper by DeRosier *et al.* (*J. molec. Biol.* **113**, 679; 1977) is therefore significant since it provides the first detailed insight into the molecular structure of cables from two different sources: the acrosomal process of horseshoe crab sperm, and extracts from sea urchin oocytes. Filaments lie parallel to each other, closely packed in a hexagonal array, and in both cases occur in association with another protein of molecular weight 55,000 (not tubulin) which crosslinks adjacent filaments. The cables are extremely regularly organised, appearing like the filtered images of actin paracrystals, and are thus well suited

Roger Craig is a Muscular Dystrophy Association postdoctoral fellow at Brandeis University.

METEOR streams are elliptical rings of dust containing a myriad of particles all moving with orbits very similar to that of the comet which ejected them as it decayed. The stream particles move through the general Solar System dust cloud which is made up of particles with random orbits. Cloud particles were once in streams but they have been perturbed from their specific orbits by interparticle collisions, close approaches to planets and radiation effects.

The Earth passes through many of these streams each year at relative velocities of between 11 and 74 km s⁻¹. Stream particles which hit the atmosphere burn up, forming visual meteor trains (shooting stars) if they are 0.1 g or more in mass, or radio meteor trains (detectable as reflectors of radar pulses) if they have masses greater than 10⁻⁷ g. Smaller stream particles are more difficult to observe. Some space-vehicle experiments have reported large increases in microparticle fluxes associated with streams. However, extrapolations of radio meteoroid fluxes to smaller masses indicate that streams contain very few of these particles. It has been suggested that the satellite borne detectors are registering micrometre-sized lunar ejecta produced by the impact of larger stream meteoroids.

Over the past few years a new method of observing meteor streams has been suggested which relies on detecting the solar radiation reflected by the orbiting stream dust particles. These should scatter radiation in a similar way to the particles in the Solar System dust cloud. The scattering from this cloud produces the zodiacal light and the streams should produce localised enhancements in this light. The problem can be approached in two ways. A. C. Levasseur-Regourd and J. E. Blamont (*Space Res.* **15**, 295; 1975) measured the zodiacal light brightness using the French satellite D2A. They found localised enhancements of brightness at specific points in the Earth's orbit. They concluded that these variations were caused by a

New look at meteor streams?

from David W. Hughes

local excess of scatters produced by the Earth's passage through a meteor stream. The photometer can point in nine directions which are all in a plane perpendicular to the Earth-Sun line. The field of view is about 3° × 3°. Sometimes the enhancement only occurred in one direction, at others in two diametrically opposite directions and in certain cases the enhancement could be detected all around the satellite. From year to year enhancements were found at the same time and in the same directions. The brightness usually increases by about 25% (that is, by between 10 and 50 S₁₀(V) units on a signal of 140 S₁₀(V) this lasting for between 1 and 17 days). (A brightness of 1 S₁₀(V) unit is equivalent to the brightness produced by having one star of visual magnitude +10.0 per square degree of the field of view or an energy flux of 1.31 × 10⁻⁹ erg cm⁻² s⁻¹ star⁻¹ A⁻³). The authors conclude that the average size of the inhomogeneity in the plane of view is about 0.15 AU. By assuming that the particles in the meteor stream have the same size and reflecting properties as sporadic zodiacal cloud particles and by measuring the spatial extent of the stream of scatters Levasseur-Regourd and R. Dumont (*COSPAR, XX Plenary meeting, Tel Aviv*, paper III C.3.2.) could estimate the ratio of the particle number density in streams to that outside. This ratio was found to be as high as 18.

W. J. Baggaley of the University of Canterbury, New Zealand, approaches the problem from another direction. He uses the known properties of the particles in meteor streams and considers how effectively they will produce zodiacal light enhancements. His theoretical calculations are given in a recent edition of *The*

David W. Hughes is a Lecturer in the Department of Physics, University of Sheffield.

Observatory (**97**, 123; 1977). Meteor stream dust is optically thin, the particle number density being so low that mutual eclipsing is rare. During an Earth-stream interception (which occurs every time we have a meteor shower) an observer looking in the direction of the true geocentric shower radiant will be observing tangentially to the stream orbit and his line of sight will intersect a large volume of dust particles. The numbers of micrometre-sized particles present have been estimated by extrapolating from particle size distribution measurements made using radar techniques. Using experimentally determined phase functions and an albedo of 0.1 Baggaley integrates along the tangent and calculates the brightness that should be observed in the geocentric radiant direction. For the Quadrantid, Perseid and Geminid streams these brightnesses are 0.57, 3.44 and 1.09 S₁₀(V) units respectively. The total sky brightnesses in these directions, caused by zodiacal light, integrated starlight and atmospheric airglow are 178, 222 and 229 S₁₀(V) units. So the streams theoretically produce an increase of at most 1.5%. The bright patches of light should be elliptical in shape because meteor streams are more dispersed in the plane of the orbit than normal to this plane (by a factor of about 10). The Quadrantids, Perseids and Geminids would produce bright patches of maximum angular extent around 10, 10 and 5 degrees respectively.

Baggaley concludes that the major annual meteor streams would not produce any observable radiant glow, assuming of course that the extrapolation of flux *versus* size data is applicable and that the measured spatial particle-number density (about 10⁻¹⁶ cm⁻³) is correct. The fact that Levasseur-Regourd and Blamont do see streams in the zodiacal light indicates that the radio meteor flux and size distribution data must be re-examined. Either that or the collisional fragmentation which perturbs stream particles into the sporadic background changes their reflecting properties.

to analysis by optical diffraction and three-dimensional reconstruction.

Since the filaments lie in a hexagonal lattice, exactly equivalent bonds could be made between each filament and its neighbours if actin subunits were to occur at azimuthal intervals of 60°. Although the non-integral helical symmetry of the actin filaments does not satisfy this condition, the symmetry is such that actin subunits present many different azimuths during one axial

repeat of the structure. By building models of filaments with the symmetries established by optical diffraction, DeRosier *et al.* have shown that the departure from 60° is small at certain points along the filament. Placing these filaments on a hexagonal lattice in the arrangement deduced from the diffraction patterns, they conclude that bonds between filaments can be made at these points if distortions are allowed. The distortions required are

small—of the same order as those discussed by Caspar and Klug for quasi-equivalent bonding between the subunits of spherical viruses (*Cold Spring Harbor Symp. quant. Biol.* **27**, 1; 1962). As pointed out by these authors, such an ordered structure need not have all identical molecules in exactly identical environments. The important point is that the lowest energy structure will have the maximum number of most stable bonds formed—and this may be

physically realised . . . by quasi-equivalent bonding of identical units.'

Support for the quasi-equivalent bonding scheme comes from the oocyte cables. Optical diffraction shows that the filaments repeat after 19 turns of the actin helix containing 41 subunits. The model predicts that 9 of the 41 subunits are appropriately oriented for quasi-equivalent bonding, and the cables reveal nine transverse stripes at the predicted axial positions in each repeat. These are thought to be due to the 55,000 molecular weight protein attaching to and crosslinking actin subunits of adjacent filaments at these points.

In the case of the acrosomal process no transverse stripes are seen. But here every actin is associated with the 55,000 component, not just those involved in crosslinking, so that all morphological subunits look alike. A bonding scheme analogous to that in the oocyte bundles has been proposed, with only those 55,000 components attached to appropriately oriented actins actually participating in crosslinking. The model seems plausible since the bond distortions in this case are less than those required in the oocyte bundles. DeRosier *et al.* have carried out a three-dimensional reconstruction of the acrosomal bundle. This reveals that the 55,000 molecular weight protein is bound to the actin subunit near the myosin binding site. While this may not be significant in a cell thought not to have myosin, it could prove relevant in other systems in controlling the interaction of actin cables with myosin.

This is the first time that actin filament cables from cells have been subjected to such analysis. The results, showing how the 55,000 molecular weight protein binds the filaments into an organised cluster, may be applicable to bundles from other sources—but with caution, since different or additional controls may prevail elsewhere.

Free calcium measurement in cells

from T. J. Lea

An EMBO Workshop on Ca Measurement in Cells was held in Oxford on 8-10 July, 1977.

It was in 1928 that H. Pollack reported that a saturated solution of alizarin sulphonate when injected into amoebae produced a shower of red crystals (Ca alizarinate) in the cyto-

plasm on the initiation of pseudopod formation. This pioneering attempt to examine cellular changes in free Ca was mentioned at the workshop during discussions of more modern methods. In a three-cornered fight between the users of photoproteins (such as aequorin), metallo-chromic indicators (such as arsenazo III) and Ca-sensitive microelectrodes, limitations of these more recently developed methods were critically examined and it became apparent that no single technique satisfied all the requirements of a good indicator of free Ca in cells.

On the evidence of results achieved, aequorin has until now led the field. Among recent advances obtained with this photoprotein was the demonstration by C. C. Ashley (University of Oxford) and colleagues of a transmitter-like action of L-glutamic acid on barnacle muscle fibres, and Ca-transients in cardiac muscle (D. G. Allen, University College London). The related indicator obelin has been used in sarcoplasmic reticulum vesicle by D. W. Yates (University of Bristol) and A. K. Campbell (Welsh National School of Medicine, Cardiff) in an attempt to measure Ca uptake by the vesicles. This method does suffer from the disadvantage of a fairly rapid consumption of the photoprotein by Ca entering the vesicles. An undoubted highlight of the meeting was the presentation by L. Jaffe (Purdue University, Indiana) on aequorin-injected eggs of the medaka, a freshwater fish. Jaffe and his colleagues succeeded in showing that immediately following fertilisation, a Ca-wave crosses a single egg (1 mm diameter) from the point of entry of the sperm and reaches the opposite side within two minutes. He concluded that because the wave is unaffected by Ca-free media, its mechanism involves a calcium-induced Ca release from some internal sites, and this idea was certainly of interest to muscle physiologists present in the audience.

The problem of quantifying the Ca measuring methods especially in relation to resting free Ca in cells was an important theme throughout the meeting. Allen produced evidence of a Ca-independent light emission from aequorin *in vitro* which he claimed gave a theoretical detection limit for free Ca in cells of 6×10^{-8} M with a practical limit of 3×10^{-7} M. Since A. Scarpa (University of Pennsylvania) and P. F. Baker (King's College, London) estimated the free Ca in squid axons using aequorin to be 2×10^{-8} M and 6×10^{-8} – 15×10^{-8} M respectively, this particular claim aroused some objections. However, in

T. J. Lea is MRC research assistant, University Laboratory of Physiology, Oxford.

general, new data given by Allen and Ashley on the shape of the aequorin–Ca calibration curve and its dependence on cytoplasmic ions, such as Na^+ , K^+ , H^+ and Mg^{2+} , should improve the accuracy of free Ca determinations made with this photoprotein.

In the opinion of I. Parker (University College, London), the absorbance indicator arsenazo III is superior to aequorin for measuring rapid transient changes in free Ca, such as those in the sarcoplasm and nerve endplate of a frog muscle fibre during a single twitch. The response time is faster, although how much faster seemed to be disputed, and the free Ca is linearly related to the absorbance change. For longer term events such as muscle tetani, he felt arsenazo to be inferior because of large movement artefacts, drift in the optical record and unexplained 'tail effects' at the end of the event. L. H. Pinto (Purdue) reported the successful use of purified arsenazo in *Limulus* photoreceptors, but found that concentrations of the dye greater than 3 mM in the cell caused changes in the membrane response to light pulses, which could be explained by arsenazo buffering of cell Ca. The strong dependence of the arsenazo absorbance on Mg^{2+} and H^+ was also emphasised by several speakers. The limit of Ca detection by arsenazo was put at $\sim 10^{-7}$ M by J. D. Owen (University of Utah, Salt Lake City).

Of the three methods, the Ca-selective microelectrode has achieved least in the field so far, but it has much to offer, particularly in the measurement of free Ca in small cells. The use of new compounds embedded in PVC membranes gives the electrodes a linear response down to 10^{-6} M free Ca, while as low as 10^{-8} M can be detected (Owen). In fact R. Meech (University of Cambridge) reported a value for the free Ca in snail neurones very similar to that of Owen's ($\sim 10^{-7}$ M corrected for Mg^{2+}) without the help of a Ca microelectrode, using instead the reversal potential of the Ca-dependent K current. Attempts to measure Ca changes during a single action potential have not so far been successful (G. Christoffersen, Copenhagen) partly because of the slow response time of these electrodes (1–2 s). □

Correction

In the article 'Meteorite research old and new' (*News and Views* 268, 691; 1977) column 3, paragraph 2, 6 lines from end should read: "Serra de Magé minerals probably did not become closed systems until 4.42 Gyr ago," not "4.52 Gyr ago."

review article

The mean lifetime of orthopositronium in vacuum

T. C. Griffith & G. R. Heyland*

Various measurements and calculations of the vacuum lifetime of ortho-positronium have given widely divergent values. There are indications that these differences are now gradually being resolved.

POSITRONIUM, the hydrogen-like atom formed when an electron and a positron are bound with a ground state binding energy of 6.8 eV, has lately become the subject of intensive study in several laboratories. It is readily formed when positrons emitted from a radioactive source are moderated to energies comparable with those of the valence electrons in atomic and molecular gases or in solid surfaces especially those of certain oxide powder grains such as MgO or SiO₂. The positronium atoms exist as either *para*-positronium, *p*-Ps, the singlet state with total spin zero or *ortho*-positronium, *o*-Ps, the triplet state with unit spin. Since the positron is the antiparticle of the electron positronium has no permanent existence; the two particles eventually annihilate one another and the probability of annihilation from the bound states can be evaluated. It is found that *p*-Ps is very short lived with a mean lifetime for decay of its ground state into two collinear γ rays, each of energy 0.511 MeV, of only 0.125 ns. Its short lifetime renders it more difficult to observe directly than *o*-Ps whose ground state, to conserve spin, has to decay into three coplanar γ rays of total energy 1.022 MeV with a mean lifetime of about 140 ns. Apart from the vacuum lifetime several other properties of positronium have been extensively investigated. After several attempts, spanning a period of about twenty years, the characteristic Lyman- α radiation from the 2P-1S transition in *o*-Ps was eventually detected by Canter *et al.*¹. There has also been a number of observations on the fine structure (or hyperfine structure) splitting of the ground state of positronium.

Theoretical background

Theoretical and experimentally-determined values of the mean lifetime for the decay of *o*-Ps in free space, which is the main subject of this article, have recently undergone considerable variation. Evaluation of this parameter involves the basic principles of quantum electrodynamics, because to high order the constituent particles interact only through the electromagnetic interaction. Its evaluation and measurement provide a clean test of quantum electrodynamics. A full formulation of the problem was presented by Strosio and Holt² and by Strosio³ and an expression was given for this mean lifetime, which includes the radiative corrections corresponding to all order terms in the fine structure constant α_f , in the form

$$\lambda_p = \lambda_p(0)[1 - (\alpha_f/\pi)(1.86 \pm 0.45)] \quad (1)$$

where $\lambda_p(0)$ is the lowest order rate given as

$$\lambda_p(0) = \frac{2\pi\alpha_f^2 m_e c^2}{h} \frac{2(\pi^2 - 9)}{9\pi} \quad (2)$$

and λ_p , the decay constant, is the reciprocal of the *o*-Ps mean lifetime. m_e is the electronic mass, c the velocity of light and h is Planck's constant.

Before these calculations only the lowest order rate had been evaluated and the initial value of 7.14 μs^{-1} given by Ore and Powell⁴ was used until the improved accuracy of α_f resulted in its upgrading to 7.2112 μs^{-1} about ten years ago. The all order calculation, Strosio³, gave $\lambda_p = (7.242 \pm 0.008) \mu\text{s}^{-1}$ which remained as the best theoretical value until the recent calculations of Caswell *et al.*⁵ who reported a much lower value of λ_p , their expression being

$$\begin{aligned} \lambda_p &= \lambda_p(0)[1 - (\alpha_f/\pi)(10.348 \pm 0.070)] \\ &= (7.0379 \pm 0.0012) \mu\text{s}^{-1} \end{aligned} \quad (3)$$

The historical sequence of events relating the above calculations to progress with the laboratory measurements, given in Table 1, has been an interesting demonstration of the importance of cross-checking and verification of results by independent research groups.

Measurement techniques—gas method

None of the measurements of λ_p carried out before those of Beers and Hughes^{6,7} and of Coleman and Griffith⁸ were sufficiently precise to warrant higher order calculations but the improvement of experimental techniques since that time has been such that measurements to an accuracy approaching 0.01% can now be contemplated. The measurements of λ_p performed before 1976 were taken using methods based on a linear extrapolation of the *o*-Ps decay rates, λ_p , in a given gas at different densities, to zero density according to the relationship

$$\lambda_p = \lambda_p + q\rho \quad (4)$$

where ρ is the gas density in amagats (1 amagat = 2.69×10^{19} molecules cm^{-3}) and q the quenching coefficient which defines

Table 1 Values for the decay rate of positronium

Year	Value of λ_p (μs^{-1})	Comment
1949	7.14	Lowest order calculation ⁴
~1970	7.2112	Upgrading of the lowest order calculation to allow for improved values of α_f
1968	7.275 ± 0.015	Measurement of Beers and Hughes ^{6,7}
1973	7.262 ± 0.015	Measurement of Coleman and Griffith ⁸
1974	7.242 ± 0.008	All order calculation of Strosio ³
1976a	7.104 ± 0.006	Precision measurement of Gidley <i>et al.</i> ⁹
1976b	7.09 ± 0.02	New measurement of Gidley <i>et al.</i> ¹⁰
1977a	7.0379 ± 0.0012	All order calculation of Caswell <i>et al.</i> ⁵
1977b	7.058 ± 0.015	Measurement of Griffith <i>et al.</i> unpublished

*Department of Physics and Astronomy, University College London, Gower Street, London WC1, UK

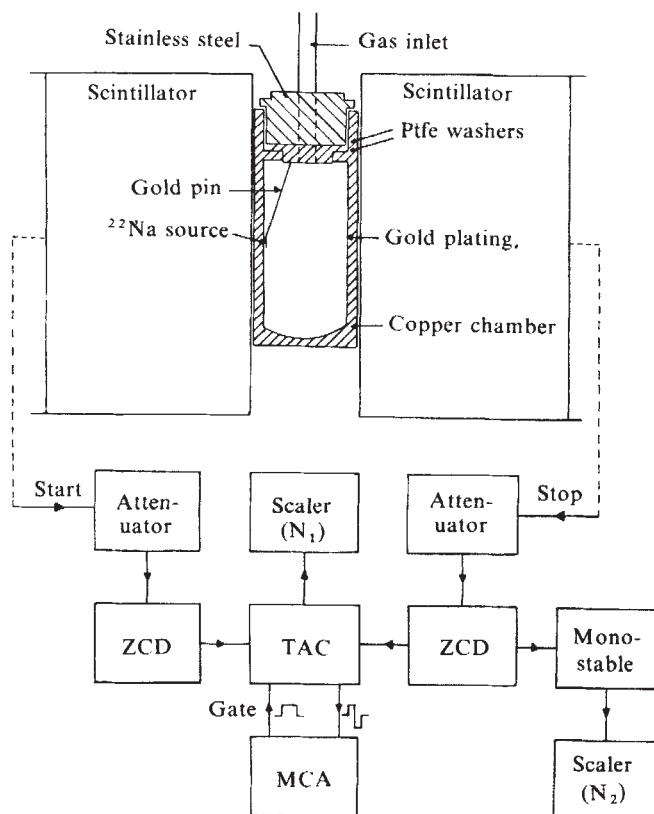


Fig. 1 Positron lifetime apparatus of Coleman *et al.*¹¹ with block diagram of the electronics. (ZCD, zero crossing discriminator; TAC, time-amplitude-converter; MCA, multi-channel analyser. N_1 and N_2 are the 'start' and 'stop' counting rates).

the increase of the decay rates, λ_p , due to collisions between the *o*-Ps atoms and gaseous molecules. The constancy of q has been established for many gases so that a linear extrapolation of equation (4) at low densities is justified.

The main features of the gas extrapolation methods are explained by reference to Fig. 1, where a schematic diagram of the lifetime apparatus of Coleman *et al.*¹¹ is given. Positrons from a ^{22}Na source were moderated in the gas contained in a small copper vessel internally gold plated to enhance back-scattering of positrons into the gas. The time interval between the emission of a positron and its subsequent annihilation is measured for a large number of events to give the positron lifetime spectrum. The 1.28 MeV γ ray emitted simultaneously with the positron is detected with one plastic scintillation counter and provides the start pulse for a timing sequence which is terminated by a pulse from the second counter which detects one of the annihilation γ rays. The lifetime spectrum of about 10^8 events accumulated in 24 hours will generally have components corresponding to the annihilation of free positrons and *p*-Ps as well as the decay of *o*-Ps. The experimental conditions for the determination of λ_p are arranged so that the component due to *o*-Ps is dominant with all the other components compressed into a narrow prompt peak near $t = 0$. This is achieved by choosing a gas, or mixture of gases, for the experiment to satisfy the following criteria: (1) high probability for formation of positronium (2) rapid thermalisation of those positrons which have not formed positronium (3) fast decay rate for the thermalised free positrons (4) small value of q (5) large molecular weight to ensure high stopping power. A mixture of ammonia-Freon 12-helium in the proportion 42%, 42% and 16% respectively, used by Griffith *et al.* (unpublished) with the above apparatus, satisfied most of these requirements and it was found that 50% of the positrons in the gas formed *o*-Ps. Even at the lowest densities the free positrons decay so rapidly that the lifetime spectrum, as shown in Fig. 2, consists of a narrow

prompt peak followed by a single exponential due to *o*-Ps decay. λ_p is derived from a distribution $D(t)$ expressed, to a good approximation, as

$$D(t) dt = [A \exp(-\lambda_p t) + B] dt \quad (5)$$

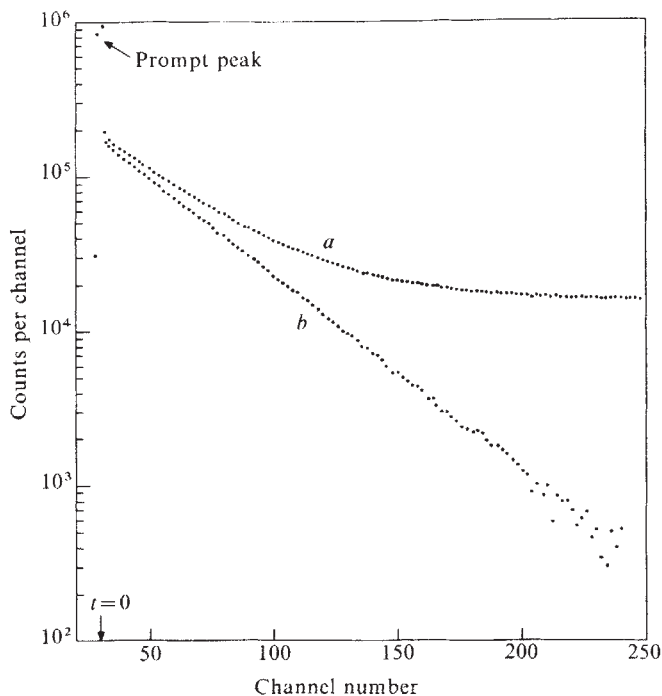
where $D(t) dt$ is the observed number of events in the time interval between t and $t + dt$ and both A and B are constants. In situations where this approximation is not valid Coleman *et al.*^{12,13} have given an exact formulation of the problem. Accurate background subtraction is very important especially for data at low densities where the signal to background, defined at A/B at $t = 0$, is low and typically varied from 31 at high density to 7 at low density in the experiment of Griffith *et al.* (unpublished).

The earlier measurements of Beers and Hughes⁶ were taken with a similar type of apparatus to that described above. They used Freon-12 alone and also a mixture of Freon-12 and argon but points below 1 amagat were not consistent with a linear plot. This non-linearity was attributed to *o*-Ps quenching at the vessel walls. Coleman and Griffith⁸ also used Freon-12 in a similar type of experiment but did not experience any difficulties at their low density points. Their ratio of A/B varied from 5 to 2 and the method of background subtraction, although more detailed than for earlier experiments, was not as accurate as that of Griffith *et al.* (unpublished). The use of the method of 'direct detection', described by Coleman *et al.*¹², to give high counting rates was a new feature in the system of Coleman and Griffith⁸. Instead of using the prompt 1.28 MeV γ ray to produce the start pulse for the timing sequence they detected the fast positrons from the ^{22}Na source in a thin plastic scintillator (of thickness 0.015 cm) as they entered the gas in the chamber. This method gave a value of ${}_o\lambda_p$ which was in agreement with that of Beers and Hughes^{6,7} and also with the calculations of Strosio and Holt².

Other methods

The method of Gidley *et al.*⁹, illustrated in Fig. 3 was also based on the principle of 'direct detection'¹². Instead of producing the *o*-Ps by moderation of fast positrons in a gas they used very fine grains of SiO_2 powder as moderator. A high yield of posi-

Fig. 2 Typical lifetime spectrum for an ammonia-freon-helium mixture at a density of 0.79 amagat units. Curve (a) represents the raw data and curve (b) the *o*-Ps component after background subtraction. For clarity only alternate data points have been plotted. $A/B = 9$, $\lambda_p = 7.396 \pm 0.007 \mu\text{s}^{-1}$. Total number of *o*-Ps events = 6×10^6 .



tronium from positrons moderated in the oxide powders MgO , SiO_2 and Al_2O_3 was first reported by Paulin and Ambrosino¹⁴ when they measured a decay constant of value close to λ_p for such powders in vacuum. This observation suggested that there was very little quenching of the *o*-Ps trapped in the interstices between the powder grains. Gidley *et al.*⁹ used grains of uniform size and assumed that the quenching was proportional to the collision rate of the *o*-Ps atoms with the grains. A parameter analogous to density in the gas experiments was defined as $\rho_s[\rho/\rho_s - \rho]$ and represented the mass per unit free volume of the powder. Accordingly λ_p was plotted as a function of

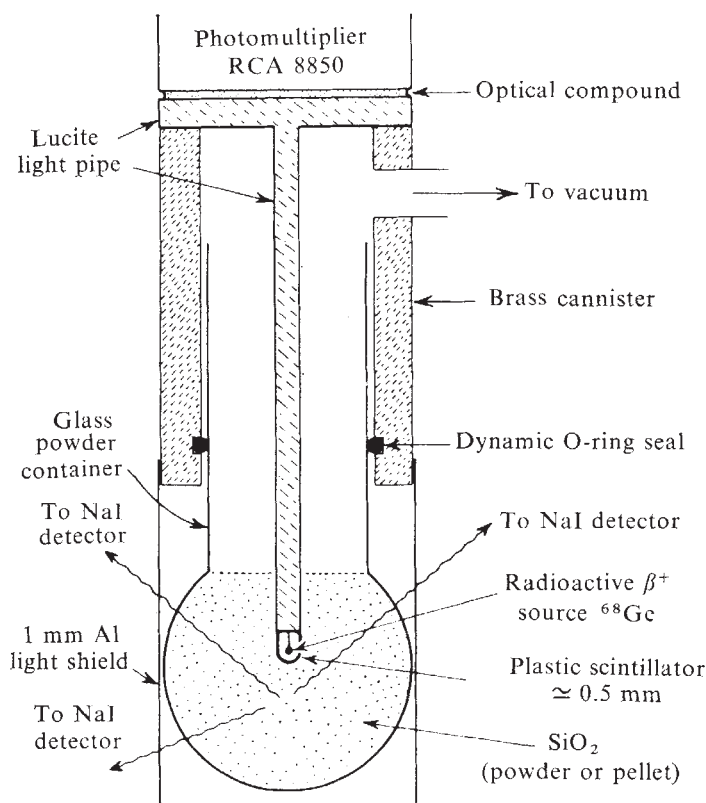


Fig. 3 Schematic diagrams of the positron "direct detection" system of Gidley *et al.*⁹ for the SiO_2 powder lifetime experiment.

$\rho/(\rho_s - \rho)$ where ρ_s is the solid aggregate density of 2.20 g cm^{-3} and ρ the powder density which was varied by compression over a range from 0.03 to 0.26 g cm^{-3} . Their results (see Fig. 4), show straight lines for two different grain sizes extrapolating to the same zero density value of $\lambda_p = 7.104 \pm 0.006 \mu\text{s}^{-1}$ which was substantially lower than all the earlier measurements and theoretical predictions. The timing sequence in this experiment was similar to that used in the gas experiments. Their efficient detection of all the positrons entering the powder through a 0.5-mm -thick plastic scintillator from a ^{68}Ge source ensured values of A/B ranging from 300 to 2,000 depending on the method adopted for producing the stop pulse from the annihilation γ rays. Analysis of the data using equation (5) was therefore accomplished to a high degree of accuracy. In common with the experiments at other laboratories precise methods were used for the time calibration of the system and a variety of tests were devised to ensure that their data was not affected by systematic errors.

The large discrepancy with previous measurements and theoretical calculations revealed by the above experiment prompted Gidley *et al.*¹⁰ to design an experiment for the direct measurement of λ_p in vacuum. The apparatus for this new experiment is illustrated in Fig. 5 and the method is based on the production of positronium from a beam of low-energy positrons as was done by Canter *et al.*¹ in their experiment to detect the positronium Lyman- α radiation. About 500 slow

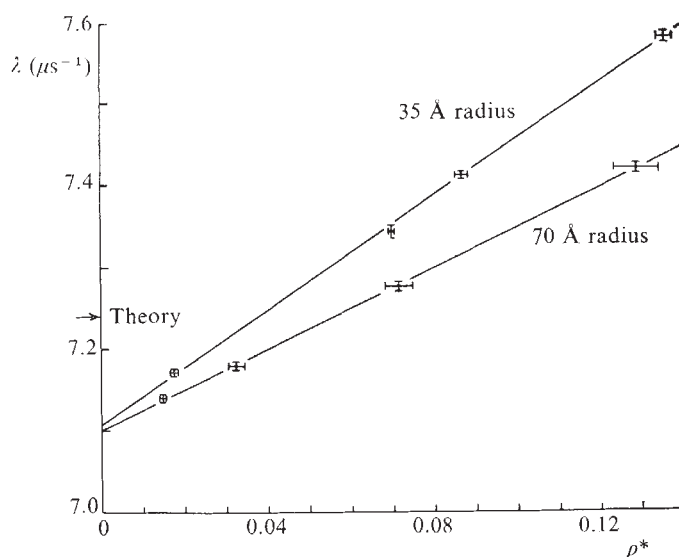


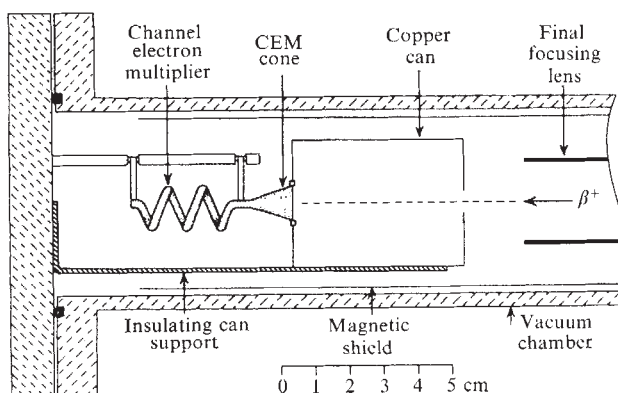
Fig. 4 O-Ps decay rates in SiO_2 powder, by Gidley *et al.*⁹, as a function of the parameter $\rho^* = \rho/(\rho_s - \rho)$ defined in the text. Curves are given for two different grain size. All samples were evacuated to 10^{-6} torr, and outgassed at 350°C for 4 h.

positrons per second, from a $46 \text{ mCi } ^{58}\text{Co}$ source followed by a gold-MgO moderator, were accelerated to 400 eV and transported down a 1 m flight path and focused through a 6-mm diameter aperture on to the MgO-coated cone of a channeltron electron multiplier. Positronium is formed on the cone with 15% efficiency and allowed to diffuse back into the evacuated copper can whose inside walls were also coated with a thin layer of MgO to reduce quenching and confine the positronium to a region for which the efficiency of detection of the annihilation γ rays is approximately uniform. The detectors were shielded from γ rays directly from the source. Backscattered positrons from the cone were stopped from entering the can by biasing the cone to -400 V and the can to -50 V . The stop pulse of the timing sequence was generated from the annihilation γ rays in the same way as for the earlier experiment⁹ but the start pulse was derived from the secondary electrons emitted simultaneously with the positronium from the channeltron cone. The lifetime spectrum was again represented by equation (5) with λ_p now replaced by λ_p measured directly and, depending on the method used to derive the stop pulse, the A/B ratio ranged from 1,500 to 9,000. Their data, of the same form as illustrated in Fig. 2, gave a mean value of $\lambda_p = 7.09 \pm 0.02 \mu\text{s}^{-1}$ which was in good agreement with their previous measurement⁹.

The meaning of recent results

The recent calculated value of $\lambda_p = 7.0379 \pm 0.0012 \mu\text{s}^{-1}$ by Caswell *et al.*⁵ strongly suggested that the measurements of

Fig. 5 Apparatus used by Gidley *et al.*¹⁰ for the direct determination of λ_p for *o*-Ps in vacuum.



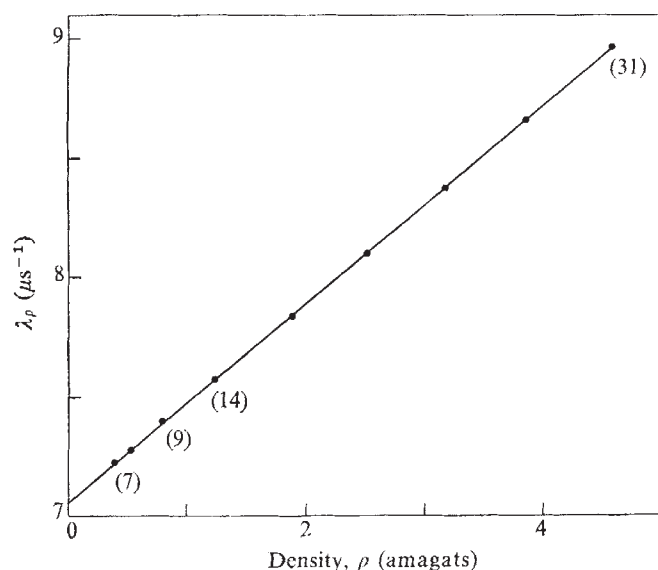


Fig. 6 The *o*-Ps decay rate λ_p as a function of gas density, ρ , for an ammonia-freon-helium mixture measured by Griffith *et al.* (unpublished). The errors are smaller than the circles enclosing the points. The numbers in parentheses represent the ratio A/B at those densities.

Gidley *et al.*^{9,10} could be closer to the true value than those obtained in the earlier experiments. This view is now reinforced by the new low value obtained in the improved gas extrapolation method of Griffith *et al.* (unpublished). Their values of λ_p , shown in Fig. 6, carry very small statistical errors and lie on a good straight line extrapolating to $7.058 \pm 0.015 \mu\text{s}^{-1}$, a value which is smaller than all the previous measurements. The gold plating on the inner walls of their vessel, by increasing the number of *o*-Ps events, has improved the A/B ratios by a factor of 5 in comparison with those of Coleman and Griffith⁸. It is probable that the very low values of A/B in the latter experiment⁸ contributed a systematic error which gave a high value of λ_p , and a similar argument may apply to the measurement of Beers and Hughes⁶.

The new value of $7.058 \pm 0.015 \mu\text{s}^{-1}$ is currently being subjected to a series of tests to establish that the measurement is

free of systematic errors. At present there is no evidence for nonlinear behaviour even at densities as low as 0.05 amagats units. This implies a negligible amount of quenching of the *o*-Ps on the gold surface. There is some uncertainty about the assignment of gas density from the pressure and temperature of mixtures of molecular gases because the virial coefficients are not accurately known, but at low density this should not be a serious error. Corrections for the mass per unit free volume have not been applied in the gas experiments because the appropriate value of ρ_s is not known. There is no evidence of deviation from a straight line at high densities which would be expected if this correction was significant.

All recent measurements of λ_p lie within the range 7.05 to 7.11 μs^{-1} and the true value is probably within these limits or lower. In common with the new gas extrapolation method the experiments of Gidley *et al.*^{9,10} may also be subject to error. In their first experiment⁹ the determination of the powder density is subject to some uncertainty and the way in which the powder packs under compression has not been investigated. Some error might also be expected in their second experiment¹⁰ due to uncertainty about the quenching of *o*-Ps at the surface of the can.

Gidley *et al.*¹⁵ have recently reported that they are also performing a gas extrapolation experiment and their preliminary values of λ_p are reported to be in agreement with their earlier values. It is clear that very accurate values of λ_p will soon be available from more than one laboratory and that the recent theoretical calculations of Caswell *et al.*⁵ will be closely tested.

- ¹ Canter, K. F., Mills, A. P. & Berko, S. *Phys. Rev. Lett.* **34**, 177-180 (1975).
- ² Strosio, M. A. & Holt, J. M. *Phys. Rev.* **10**, 749-755 (1974).
- ³ Strosio, M. A. *Phys. Rep.* **22**, C, 217-277 (1975).
- ⁴ Ore, A. & Powell, J. L. *Phys. Rev.* **75**, 1696-1699 (1949).
- ⁵ Caswell, W. E., Lepage, G. P. & Sapirstein, J. *Phys. Rev. Letts.* **38**, 488-491 (1977).
- ⁶ Beers, R. H. & Hughes, V. W. *Bull. Amer. Phys. Soc.* **13**, 633 (1968).
- ⁷ Hughes, V. W. *Phys. 1973, Plenarvortrag Physik-ertug 37th* 123-155 (Physik Verlag, Weinheim, 1973).
- ⁸ Coleman, P. G. & Griffith, T. C. *J. Phys. B: Atom. Molec. Phys.* **6**, 2155-2161 (1973).
- ⁹ Gidley, D. W., Marko, K. A. & Rich, A. *Phys. Rev. Lett.* **36**, 395-398 (1976).
- ¹⁰ Gidley, D. W., Zitzewitz, P. W., Marko, K. A. & Rich, A. *Phys. Rev. Lett.* **37**, 729-732 (1976).
- ¹¹ Coleman, P. G., Griffith, T. C., Heyland, G. R. & Killeen, T. L. *Appl. Phys.* **3**, 271-273 (1974).
- ¹² Coleman, P. G., Griffith, T. C. & Heyland, G. R. *J. Phys. E*, **5**, 376-378 (1972).
- ¹³ Coleman, P. G., Griffith, T. C. & Heyland, G. R. *Appl. Phys. S*, 223-230 (1974).
- ¹⁴ Paulin, R. & Ambrosino, G. *J. Phys. (Paris)* **29**, 263-270 (1968).
- ¹⁵ Gidley, D. W., Rich, A., Zitzewitz, P. W. & Paul, D. A. L. *Bull. Amer. Phys. Soc.* **22**, 586 (1977).

articles

Positions of galactic X-ray sources: $20^\circ < l^{\text{II}} < 55^\circ$

R. E. Doxsey, K. M. V. Apparao*, H. V. Bradt, R. G. Dower & J. G. Jernigan

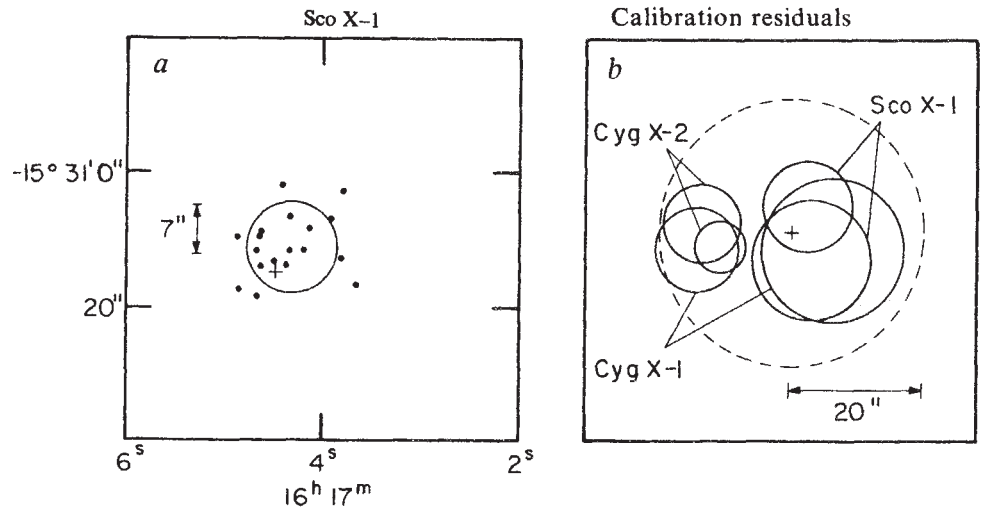
Department of Physics and Center for Space Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Precise positions, determined with data from the SAS-3 X-ray observatory, are presented for eight galactic plane X-ray sources (Aql X-1, Ser X-1, 3U1956+11, 3U1822-00, 3U1915-05, A1845-02, A1850-08, A1905+00). Error radii for the positions range from 20 to 50 arc s. Previously proposed optical identifications of three of the sources (Ser X-1, 3U1956+11, A1850-08) are supported by these results. Three (Ser X-1, A1905+00, 3U1915-05) have been identified as X-ray burst sources.

THE rotating modulation collimator (RMC) system on the SAS-3 X-ray observatory has been used to carry out a systematic survey of the galactic plane ($|b^{\text{II}}| \lesssim 10^\circ$). The aim of the survey is to identify galactic X-ray sources by the precise measurement of their positions (error radii $< 60''$). A few preliminary results based on small amounts of quick-look data have been previously reported¹. The availability of production data and a more complete calibration of the instrument now allows a more thorough, sensitive,

*Permanent address: Tata Institute of Fundamental Research, Bombay, India.

Fig. 1 *a*, Sco X-1 calibration of 2.3' collimator. Each dot represents the position determined for Sco X-1 from one orbit of data. The circle is centred at the average position and has a radius equal to the r.m.s. scatter of the points (7"). + indicates the position of the optical counterpart. *b*, Calibration residuals for the 2.3' collimator. The data for each calibration observation has been analysed and plotted as in *a*. The circles representing the r.m.s. scatter of points about the average positions have been overlayed with the true position used as a registration point. The r.m.s. scatter of the average positions from the true position is 12". The 20" dashed circle represents the error radius for a source position limited only by systematic errors.



and more accurate survey than previously possible. We discuss here the instrument calibrations and present results of the analysis for the region $20^\circ < l^h < 55^\circ$. This region has been previously surveyed with the UHURU (ref. 2) and Ariel V (ref. 3) satellites.

The SAS-3 RMC system consists of two independent modulation collimators^{4,5}. One has a periodic triangular response function with a FWHM of 2.3', the other, 4.5'. Both modulation collimators have $12^\circ \times 12^\circ$ FWHM coarse collimators to limit the field of view. Proportional counters sensitive in the range 2–11 keV are used as X-ray detectors. The centre of the overall field of view is aligned with the spacecraft spin axis. RMC survey data are taken with the satellite rotating uniformly about its axis at ~ 1 –2 revolutions per 95-min orbit. Star trackers provide data from which the motion of the spacecraft relative to the celestial sphere can be reconstructed to an accuracy of 5–10".

Accuracy of position determinations

The calibration of the RMC system was determined from observations of several bright, optically identified X-ray sources; in particular Sco X-1, Cyg X-1, and Cyg X-2. These observations have been made periodically throughout the lifetime of the satellite and are designed to cover the range of parameters normally encountered (spin rate and direction, temperature, and location of source in field of view). These data have also been used to investigate the short and long term stability of the star tracker-collimator system.

Two orientation angles and the collimator periodicity define completely the transmission response of a modulation collimator over its entire field of view. The two angles required are the rotational orientation of the collimator about the spacecraft spin

axis and the phase of the spin axis perpendicular to the periodic transmission bands. If the true celestial position of the source in the field of view is known, then a set of linearised least square fits to the data can be used to determine these angles. The data from two observations of Sco X-1 (19–20 May 1975 a d 9–10 July 1975) were used in this way. Each satellite orbit of data was independently analysed and fit to determine the three parameters. The average value of each parameter for each collimator was then fixed for use in the subsequent analyses.

X-ray source positions are obtained from a computation of the cross-correlation between the data and the expected response for a two-dimensional grid of sky points⁶. A grid separation of $\sim 1'$ is used to compute a 'map' of the central region ($15^\circ \times 15^\circ$) of the field of view. Precise positions are obtained from a high resolution map ($\sim 1''$ grid) in the local region of a source. Subtraction of the Bessel function patterns of the bright sources often reveals fainter sources, not noticeable in the original map⁶.

The two Sco X-1 observations were used to test the mapping algorithm and the reproducibility of the position determination on a short (~ 1 d) time scale. Sco X-1 is sufficiently bright so that the effect of counting statistics on the measured position is negligible ($< 1''$) for a one-orbit exposure. The correlation map technique was applied independently to each orbit of data. The r.m.s. scatter of positions relative to the average position was 7" for one Sco X-1 observation (Fig. 1a) and 9" for the other. We believe that this scatter of the positions is due to small random errors in the star tracker attitude solution since the orbit-to-orbit variations for the two collimators are highly correlated. The offset (5") between the average position and the optical counterpart reveals a systematic error.

Fig. 2 Line drawings of regions containing X-ray sources reported in this paper. U, UHURU error boxes²; A, Ariel V error boxes³; S, SAS-3 error circles. In cases where two SAS-3 error circles are shown, the larger is the previously reported quick-look result.

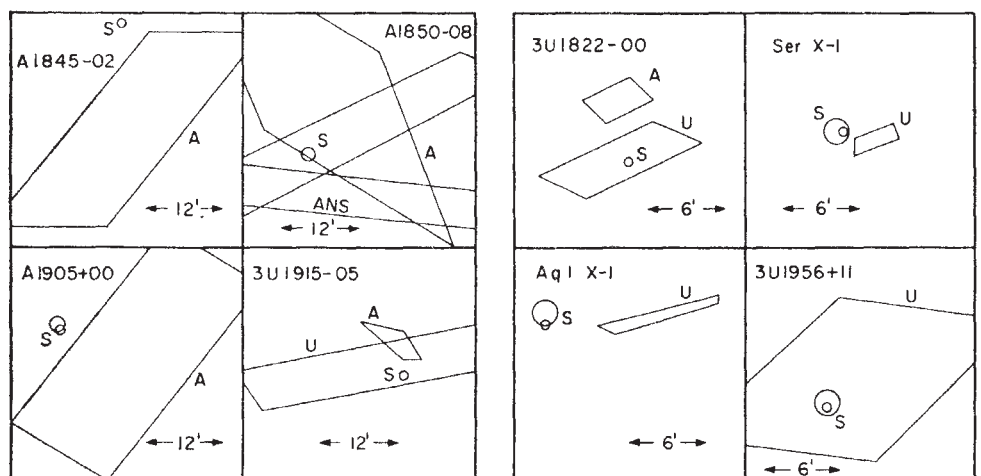


Table 1 Celestial positions

SAS-3 designation	Other designations	Position α (1950)	δ	l^{II} b^{II}	Error radius (90%)	Flux density* (2–11 keV)	Comments
2S1822–000	3U1822–00 A1822+00	18h 22min 48.5s 275.7021 ^o	–00°02'24" –0.0400 ^o	29.9 ^o 5.8 ^o	20"	30 μ Jy	
2S1837+049	Ser X–1 3U1837+04	18 37 29.8 279.3742	04 59 23 4.9897	36.1 4.8	20	250	Optical candidate ⁸ Burst source MXB1837+05 (refs 10–12)
2S1845–024	A1845–02	18 45 41.1 281.4213	–02 28 37 –2.4769	30.4 –0.4	35	11	
2S1850–087	A1850–08	18 50 21.6 282.5900	–08 45 36 –8.7600	25.4 –4.3	50	5	Globular cluster NGC6712
2S1905+000	A1905+00	19 05 54.9 286.4788	00 05 37 0.0936	35.0 –3.7	35	10	Burst source MXB1906+00 (refs 11, 18)
2S1908+005	Aq1 X–1 3U1908+00	19 08 43.6 287.1817	00 30 05 0.5014	35.7 –4.1	20	45	
2S1916–053	3U1915–05 A1916–05	19 16 08.5 289.0354	–05 19 51 –5.3308	31.4 –8.5	35	20	Burst source ^{20,24}
2S1957+115	3U1956+11 A1956+11	19 57 02.9 299.2621	11 34 14 11.5706	51.3 –9.3	20	24	Optical candidate ²¹

*Averaged over the observation and accurate to $\sim 10\%$. We quote the flux density at 5.2 keV of an incident power-law energy spectrum with slope $\alpha_{\text{pl}} 0.8$ (for example, the Crab nebula²³). 1.0 μ Jy corresponds to 2.2×10^{-11} erg s^{–2} (2–11 keV). $I = 1,060 \mu$ Jy and 1.0 RMC count s^{–1} = 2.7 μ Jy.

The systematic errors were further evaluated with a comparison of the average positions determined for Cyg X-1 and Cyg X-2 and the true positions (Fig. 1b). We find an r.m.s. scatter of 12" in the difference between true and measured positions for both collimators. This is larger than can be accounted for either by counting statistics or the random attitude solution error. The errors are not consistent with a correlation between the calibration parameters and temperature, satellite age, or spin rate. There is no set of values for the calibration parameters which will reduce this scatter. Therefore, we adopt the calibration parameters determined from the Sco X-1 data for the analysis of the survey data.

The error radii for X-ray source locations are determined from an evaluation of four sources of error: counting statistics, random aspect errors, systematic errors, and errors resulting from the subtraction of bright sources. Each of these errors is modelled as a Rayleigh distribution (two-dimensional Gaussian). The contribution due to counting statistics is $\sigma_1 = 1.7 \delta N_T^{1/2} N_S^{-1}$, where δ is the FWHM of the collimator (2.3' or 4.5'), N_T is the total number of counts recorded, and N_S is the number of X rays detected from the source. The factor 1.7 is calculated from the specifics of the cross-correlation computation and has been verified empirically. The contribution due to systematic errors is $\sigma_2 = 12''/2^{1/2} = 8.5''$ (for a Rayleigh distribution, $\sigma = \theta_{\text{r.m.s.}} 2^{-1/2}$). The random aspect error is $\sigma_3 = 10''(2n)^{-1/2}$, where n is the number of orbits of data in the observation. Since n is generally greater than 10, this term is usually negligible. The error resulting from the subtraction of a bright source is $\sigma_4 = 0.6 \delta/f$, where f is the ratio of the intensity of the faint source to the intensity in the same region of the residual ripples from the bright source after subtraction. This ratio must be evaluated for each source; in most cases $f > 30$ and $\sigma_4 < 5''$.

The 90% confidence error radius for the combined error is then $E_r = 2.15 (\sigma_1^2 + \sigma_2^2 + \sigma_3^2 + \sigma_4^2)^{1/2}$. This radius ranges from $\sim 60''$ for a source near the detection threshold to $\sim 20''$ for a source limited only by systematic errors. A source position and its associated error are calculated for each collimator from all the data taken with the satellite spin axis at one location relative to the source.

We usually obtain several pairs of independent position determinations by observing each source from a number of different spin axis locations. The quoted position is the weighted average of the positions determined for each observation. The quoted error radius is representative of the error radii computed for the

individual observations. An empirical error radius, E_r' , is calculated from the scatter of the determined positions. We have found so far good agreement between E_r' and our chosen error radius. If, occasionally, we do not find them to be comparable, we will adopt the larger value.

Results

The region of the galactic plane between longitudes 20° and 55° was observed 11–16 July 1975, immediately after one of the observations of Sco X-1, and also on 13 September 1975. The spin axis was pointed for 1 d at each of six 'targets' in the region. The overall

Table 2 Stellar astrometry and magnitudes

Source	Star	m_{pg}^*	Position (1950) [†] α δ
2S1822–000	1	16.9	18h 22min 46.7s
	4	16.5	–00°04' 26"
	5		18 22 54.0 –00 03 03
2S1837+049 (Ser X–1)	D	18.5 $\ddagger$	
	10	17.9	
	11	15.1	18 37 24.4 05 00 47
2S1845–024	2	16.2	18 45 41.6 –02 28 34
	3	18.6	18 45 43.3 –02 29 10
2S1905+000	3	18.9	
	12	17.4	
	27	14.8	19 05 52.1 00 05 01
2S1908+005 (Aq1 X–1)	3	17.9	
	13	12.7	19 08 42.7 00 31 07
	14		19 08 46.1 00 28 33
2S1916–053	2	17.9	
	19	12.9	19 16 07.4 –05 21 06
	20		19 16 13.0 –05 18 51
2S1957+115	M	19.0 $\ddagger\ddagger$	
	8	17.2	
	10		19 56 55.4 11 34 01
	11	16.0	19 57 07.6 11 34 30

*Estimated from stellar diameters⁷ to ~ 0.5 mag.

[†]Precise to $\lesssim 2''$.

[‡] m_{B} from other work^{8,21}.

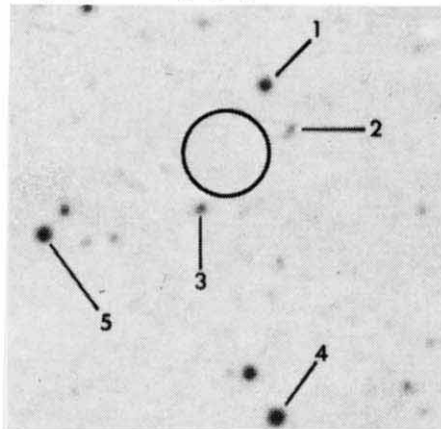
field of view was larger than the separation between targets, so each source was detectable in the data from at least two independent observations. Earth blockage, South Atlantic anomaly penetration, and partial loss of daytime star tracker data reduced the usable data in each of these observations to $\sim 30,000$ s.

A total of eight previously known X-ray sources were detected in this region. The positions determined for these sources are given in Table 1. Error circle radii were determined as discussed above. The contribution to the total error radius from the random aspect and

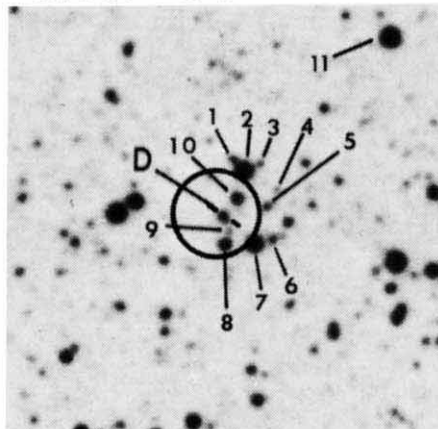
source subtraction errors was negligible for all of these sources. In all cases, the scatter in the positions determined from independent observations was less than the computed error radius. Source intensities (2–11 keV) are averaged over the one day observation and are accurate to $\sim 10\%$. The positions are plotted relative to previous results in Fig. 2. Finding charts for each of the sources are provided in Fig. 3. Table 2 contains celestial positions and magnitudes estimated from image diameters⁷ for several stars in each finding chart.

Fig. 3 Finding charts for the SAS-3 positions. The charts are taken from the Palomar Sky Survey blue plates (National Geographic Society). North is up and east is to the left. The indicated scale factor applies to all charts. Stars are numbered to ease communication between observers. Letter designations indicate proposed identifications (see text).

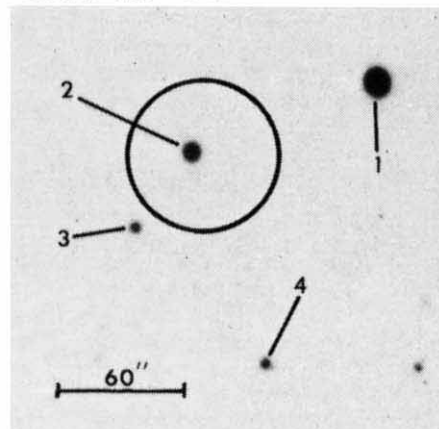
2S1822-000



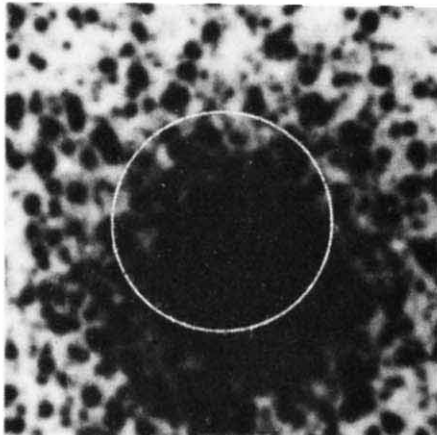
2S1837+049



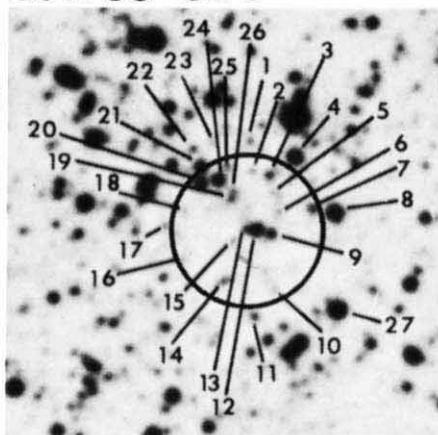
2S1845-024



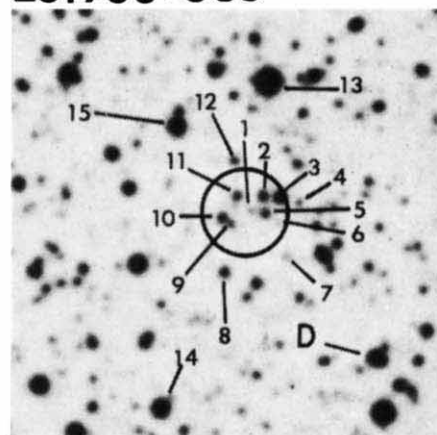
2S1850-087



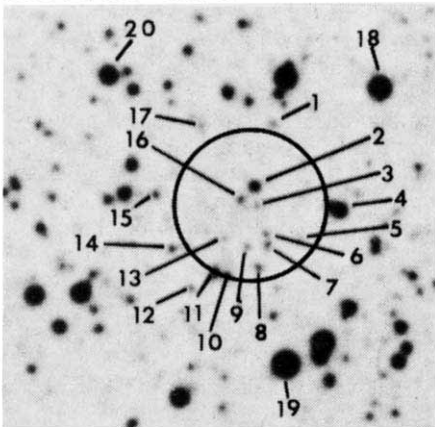
2S1905+000



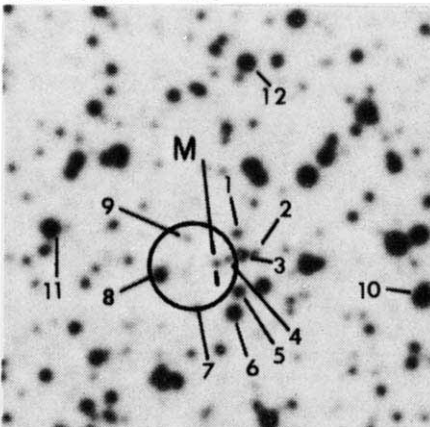
2S1908+005



2S1916-053



2S1957+115



There are 11 sources detected in the UHURU and Ariel V surveys of this region which we have not seen. If these sources were at their catalogued intensities, then only one, 3U1901+03, should have been clearly above our detection threshold. Another one, 3U1912+07, would have been above our threshold if it were at the intensity reported in the 3U catalogue², but below our threshold at the intensity reported by Seward³.

The current status, with regard to the identification of these objects, is as follows:

3U1822-00: there have been no proposed candidates for this source. There are no stars visible on the Palomar Sky Survey blue plate in the error circle and only a few on the red plate.

Ser X-1: Davidsen⁸ proposed a candidate for Ser X-1 based on the SAS-3 quick-look results⁹. The object is a faint ($B \approx 18.5$) star with an ultraviolet excess. It is $\sim 4''$ from the centre of our refined error circle. Ser X-1 is also a source of X-ray bursts¹⁰⁻¹², the first burst source for which a stellar counterpart has been proposed.

A1845-02: There have been no proposed candidates for this source. The source lies in a heavily obscured region. There is only one star visible on the Palomar Sky Survey inside the error circle.

A1850-08: The globular cluster NGC6712 was proposed by Seward³ *et al.* as a possible counterpart for A1850-08. Cominsky *et al.*¹³, using UHURU data, and Grindlay *et al.*¹⁴, using ANS data, improved the position determination of this source. In both cases, NGC6712 was consistent with the data, but field objects could not be ruled out. Our position for this source clearly identifies it with the cluster. The centre of the cluster is $\sim 30''$ from our best position, well inside the error circle. Of the known variable stars in the cluster, two long period variables, V2 and V8, lie within the error circle. Also lying within the error circle is V17, which was seen to vary by Harwood¹⁵, but not by Sandage¹⁶ or Rosino¹⁷.

A1905+00: there are currently no proposed candidates for this object. The source has been identified as an X-ray burst source^{11,18}.

Aql X-1: a candidate for Aql X-1 was proposed by David-

sen¹⁹ based on the UHURU error box. This candidate is ruled out by our results.

3U1915-05: The star 26f Aql, suggested as a possible counterpart in the 3U catalogue² is not consistent with our results. This source was first proposed as a burst source candidate by Becker *et al.*²⁰ based on OSO-8 observations (error box area $\sim 13 \text{ deg}^2$) and has been confirmed with recent SAS-3 observations (error box area $\sim 0.1 \text{ deg}^2$ (ref. 24)).

3U1956+11: Margon *et al.*²¹ have proposed a candidate for this source based on the SAS-3 quick-look results²². It has a B magnitude of 19.0 with a spectrum similar to that of Sco X-1. It lies $10''$ from our present refined position.

We thank the staffs of the Center for Space Research at M.I.T. and the Goddard Space Flight Center. Stellar coordinates were measured at facilities of the Kitt Peak National Observatory. This work was supported in part by the US NASA. This is the second in the series of articles on positions of galactic X-ray sources with SAS-3 (ref. 1 being the first).

Received 5 July; accepted 2 August 1977.

¹ Bradt, H. V. *et al.* *Nature* **269**, 21-25 (1977).

² Giacconi, R. *et al.* *Astrophys. J. Suppl.* **27**, 37-64 (1974).

³ Seward, F. D., Page, C. G., Turner, M. J. L. & Pounds, K. A. *Mon. Not. R. Astr. Soc.* **175**, 39P-46P (1976).

⁴ Doxsey, R. *et al.* *Astrophys. J. Lett.* **203**, L9-L12 (1976).

⁵ Schnopper, H. W. *et al.* *Astrophys. J. Lett.* **210**, L75-L77 (1976).

⁶ Schnopper, H. W. *et al.* *Astrophys. J. Lett.* **161**, L161-L167 (1970).

⁷ Liller, M. H. & Liller, W. *Astrophys. J. Lett.* **199**, L133-L135 (1975).

⁸ Davidsen, A. *I.A.U. Circ.* No. 2824 (1975).

⁹ Doxsey, R. *I.A.U. Circ.* No. 2820 (1975).

¹⁰ Swank, J. H., Becker, R. H., Pravdo, S. H. & Serlemitsos, P. J. *I.A.U. Circ.* No. 2963 (1976).

¹¹ Li, F. & Lewin, W. H. G. *I.A.U. Circ.* No. 2983 (1976).

¹² Li, F. K. *et al.* *Mon. Not. R. Astr. Soc.* **179**, 21P-25P (1977).

¹³ Cominsky, L., Forman, W., Jones, C. & Tananbaum, H. *Astrophys. J. Lett.* **211**, L9-L13 (1977).

¹⁴ Grindlay, J. E. *et al.* *Astrophys. J. Lett.* **212**, L67-L71 (1977).

¹⁵ Harwood, M. *Ann. Sterrewacht Leiden* **21**, 387 (1962).

¹⁶ Sandage, A., Smith, L. L. & Norton, R. H. *Astrophys. J.* **144**, 894-902 (1966).

¹⁷ Rosino, L. *Astrophys. J.* **144**, 903-915 (1966).

¹⁸ Lewin, W. H. G. *et al.* *Mon. Not. R. Astr. Soc.* **177**, 93P-100P (1976).

¹⁹ Davidsen, A., Malina, R. & Bowyer, S. *Astrophys. J.* **203**, 448-454 (1976).

²⁰ Becker, R. H. *et al.* *Astrophys. J.* (in the press).

²¹ Margon, B., Thorstensen, J. & Bowyer, S. *Bull. Am. Astr. Soc.* **9**, 330 (1977).

²² Bradt, H., Doxsey, R., Jernigan, J. G. & Spada, G. *Bull. Am. Phys. Soc.* **21**, 544 (1976).

²³ Toor, A. & Seward, F. D. *Astr. J.* **79**, 995-999 (1974).

²⁴ Lewin, W. H. G., Hoffman, J. A. & Doty, J. *I.A.U. Circ.* No. 3087 (1977).

Supernovae, grains and the formation of the Solar System

James M. Lattimer

Department of Astronomy, University of Illinois, Urbana, Illinois 61801

David N. Schramm & Lawrence Grossman

The Enrico Fermi Institute, University of Chicago, Chicago, Illinois 60637

The observed ^{26}Mg and ^{16}O anomalies in meteorites can be consistently understood if a supernova occurred within a few million years of the condensation of the Solar System. Grains condensing in the ejecta from this supernova may be an integral aspect of this process.

THE detection^{1,2} of excess ^{26}Mg which correlates with the Al/Mg ratio in a Ca-rich inclusion in the C3 chondrite Allende offers strong evidence that a nucleosynthetic event preceded the formation of the Solar System by at most a few million years. Two explanations for the production of ^{26}Al , which decayed to form the observed excess ^{26}Mg , have been presented. (1) An irradiation in the early Solar System³ could synthesise the observed amount of ^{26}Al throughout a solar nebula of $1 M_{\odot}$, but the energy required is greater than the binding energy of the Sun. This seems unreasonably large. In addition, excess amounts of other rare nuclides such as ^{40}K and ^{50}V , as well as neutron-induced isotopic anomalies in elements such as Gd, would be produced. No such anomalies have been observed to date. (2) One or more super-

novae, which could produce ^{26}Al in either explosive carbon burning or in a high temperature carbon burning shell source immediately preceding the explosion, occurred near the protosolar cloud⁴. This supernova would have had to occur within a few ^{26}Al decay half lives (7.2×10^5 yr). The fact that the protosolar cloud condensed within such a short time of this supernova may not be accidental; the supernova trigger hypothesis of the formation of the Solar System has been considered by several authors (ref. 4 and S. Margolis, in preparation). We present here the results of our supernova grain condensation calculations and relate them to the hypothesis that a 'last event' supernova was indeed related to the formation of the Solar System and thus might have created the observed isotopic anomalies in magnesium, oxygen, neon and xenon.

Four components of presolar material are envisaged for the formation of the Solar System. The first two, interstellar gas and dust, have been enriched by supernova and nova debris (gas and possibly dust) and stellar winds, among other processes, throughout galactic history. The average composition of the dust may be chemically and isotopically different from the gas. The last two

Table 1 Isotopic anomalies and condensing minerals in supernova ejecta

Supernova zone	Non-solar isotopic abundance peculiarities	Major condensible species at 10^{-8} atm which also condense in the solar nebula*	
(H)	$^{13}\text{C}(+)$, $^{15}\text{N}(\pm)$, $^{17}\text{O}(+)$	Al_2O_3	C , TiC , SiC , Fe_3C
(He)	$^{13}\text{C}(-)$, $^{15}\text{N}(\pm)$, $^{17}\text{O}(-)$ $^{18}\text{O}(\pm)$, $\text{Mg}(n)$, $\text{Si}(n)$ $\text{S}(n)$, $\text{Ca}(n)$, $\text{Ti}(n)$, $\text{Fe}(n)$	$\text{Ca}_2(\text{Al}_2, \text{MgSi})\text{SiO}_7$ MgAl_2O_4 CaTiO_3 $\text{CaMgSi}_2\text{O}_6$	AlN , CaS , Fe Al_2O_3 , MgAl_2O_4 $\text{CaMgSi}_2\text{O}_6$
(C)	$^{12}\text{C}(+)$, $^{16}\text{O}(+)$, $^{25,26}\text{Mg}(\pm)$ $^{26}\text{Al}(+)$, $^{29}\text{Si}(+)$, $^{30}\text{Si}(\pm)$ $\text{S}(n)$, $\text{Ca}(n)$, $\text{Ti}(n)$, $\text{Fe}(n)$		C , TiC , SiC , Fe CaS , Al_2O_3 MgAl_2O_4 , CaTiO_3
(O)	$^{16}\text{O}(+)$, $^{24}\text{Mg}(+)$, $^{28}\text{Si}(+)$ $^{36}\text{S}(-)$, $^{40,42}\text{Ca}(+)$ $^{46}\text{Ti}(+)$, $\text{Fe}(n)$	$(\text{S} + \text{Si})/\text{O} < 1$ Al_2O_3 , CaTiO_3 $\text{Ca}_2(\text{Al}_2, \text{MgSi})\text{SiO}_7$ Fe , $\text{CaMgSi}_2\text{O}_6$, CaS	$(\text{S} + \text{Si})/\text{O} > 1$ CaS , Fe , Al_2O_3 $\text{Ca}_2(\text{Al}_2, \text{MgSi})\text{SiO}_7$ $\text{CaMgSi}_2\text{O}_6$
(Si)	$^{32}\text{S}(+)$, $^{40}\text{Ca}(+)$	Fe , CaS	

(-) Possible depletion relative to other stable isotopes and solar abundances; (+), possible enhancement relative to other stable isotopes and solar abundances; (n), possible enhancement of neutron-rich isotopes due to neutron capture. ($\pm$), Indicates uncertainty in the production relative to other stable isotopes.

*Solar nebula conditions: 10^{-3} atm pressure and either $T > 1,430$ K, $\text{C/O} < 1$ or $T > 1,300$ K, $\text{C/O} > 1$ and otherwise solar¹² composition.

components, 'last-event' supernova gas and dust, may be mixed into the protosolar cloud when the shock wave from this supernova collides with the cloud and initiates its collapse. We note here, in reference to the problem of producing observable isotopic anomalies, that it may be easier for the 'shrapnel-like' grains from the supernova to penetrate to the interior of the cloud than for supernova gas. This gas, part of which mixes by Rayleigh-Taylor 'interfingering' at the interface between the expanding supernova shock front and the protosolar cloud, may mostly just pass around the exterior of the collapsing presolar nebula. In addition, the time required for injected supernova gas to completely mix with nebular gas may be so short that production of non-radiogenic isotopic anomalies with supernova gas alone might be precluded.

The currently accepted model⁶ of presupernova stars pictures them to be composed of several layers. The outer layer has roughly 'solar' composition, while successive interior shells have compositions corresponding to the products of static hydrogen, helium, carbon, oxygen and silicon burning, respectively. The supernova occurs when the presupernova core collapses, and a shock wave explosively burns and ejects the outer layers of the star. In some stars, however, the shock wave may not cause significant explosive processing. Because the most complete estimates of the composition of supernova ejecta are explosive burning calculations, we assume that these ejecta contain zones composed, successively, of the products of explosive hydrogen, helium, carbon, oxygen and silicon burning. These zones are hereafter referred to as the (H), (He), (C), (O) and (Si) zones.

Table 1 shows, for each explosive burning zone, the isotopic compositions⁷⁻⁹ of the major elements that are decidedly non-solar. Entries are of three types: first, the oversynthesis of one or more isotopes of an element, relative to the others, compared with solar isotopic abundances (namely ^{32}S , ^{33}S , ^{40}Ca and ^{42}Ca in the (C) zone); second, the survival of only one isotope of an element (namely ^{16}O and ^{24}Mg in the (O) zone); or, third, the enhancement of the neutron-rich isotopes of an element relative to solar abundances due to neutron capture during explosive burning or the static helium burning¹⁰ phase that preceded explosive carbon and oxygen burning (namely Ti in the (He) zone).

To discover what minerals could condense in supernova ejecta, equilibrium condensation calculations for each of these zones have been performed¹¹. The results for 10^{-8} atm pressure and different C/O and (S + Si)/O ratios are also shown in Table 1. We have listed, for reasons discussed below, only those minerals that are also condensible at 10^{-3} atm, assuming either that $T > 1,430$ K and $\text{C/O} < 1$ or $T > 1,300$ K and $\text{C/O} > 1$, and otherwise solar¹² abundances. The calculations are not very sensitive to pressure

changes. The most important compositional parameter is the C/O ratio, or, in the (Si) zone, the (S + Si)/O ratio. Grains condensed from a given zone will have an isotopic composition characteristic of that zone and are hence capable of carrying that composition into the presolar nebula. But it is not at all certain that grains can form in expanding supernova ejecta. Preliminary estimates¹¹ suggest that grain condensation is only possible if the shock wave that explosively burns and ejects the matter is relatively weak. If grains do form these results indicate their probable compositions.

As the collapse of the presolar nebula proceeds, the pressure and temperature rise. If isotopic anomalies are to be carried into the Solar System in grains, these grains must be able to survive, that is, their composite minerals must be condensible in this physical environment. We are primarily interested in Ca-rich inclusions of C3 chondrites, which seem to have formed in a nebular region that had a maximum pressure of about 10^{-3} atm and a roughly solar¹² composition. The maximum temperature, T_{max} , that this region reached must have been greater than the condensation temperature of forsterite¹¹, 1,430 K, because the bulk chemical compositions of the Ca-rich inclusions are similar to those calculated for the pre-forsterite condensate and dramatically different from the post-forsterite composition. If $T_{\text{max}} > 1,742$ K, no major O-bearing mineral could survive. But corundum (Al_2O_3), perovskite (CaTiO_3), melilite [$\text{Ca}_2(\text{Al}_2, \text{MgSi})\text{SiO}_7$], spinel (MgAl_2O_4) and diopside ($\text{CaMgSi}_2\text{O}_6$) could survive if $T_{\text{max}} < 1,742, 1,681, 1,625, 1,530$ and $1,435$ K, respectively. Because all these minerals could condense in at least two supernova zones, isotopic anomalies in the elements O, Mg, Si, Ca and Ti may eventually be observable in Ca-rich inclusions.

Excess ^{16}O (ref. 13) and ^{26}Mg have been observed in these inclusions and could have been carried into the solar nebula in grains condensed in the (C) zone (Table 1). In addition, excess ^{16}O is also produced in the (O) zone. Lee *et al.*² found that there is a positive linear correlation between the excess ^{26}Mg and the Al/Mg ratio, that is, the data define an Al-Mg isochron. Clayton *et al.*¹³ found the amount of excess ^{16}O to be fairly constant within a given phase, even from samples taken from different inclusions. Both observations may be easier to understand on the basis of a gaseous rather than a crystalline source of these anomalies. But textural¹⁴ and trace element¹⁵ evidence imply that these inclusions were at least partially melted during or after their formation. Since diffusion in silicate melts is more rapid than in crystalline silicates, all phases crystallising from or equilibrating with a melt would tend to have the same isotopic composition, thus obliterating any previous distribution that could be used to ascertain the source of these anomalies. Those phases which did not melt would be

expected to have different isotopic compositions. Experiments¹⁶ have shown that spinel and pyroxene have higher minimum melting temperatures in this system than melilite and anorthite. Observations¹³ show that the spinel and pyroxene of Ca-rich inclusions have greater concentrations of excess ¹⁶O than melilite, by about a factor of four. This could be explained by a partial melting of the inclusions at a time when there was less excess ¹⁶O in the ambient gas than in the inclusion. On the same grounds, one could expect deviations from the Mg–Al isochron to occur. But, the higher melting point phases (spinel, pyroxene) have such small Al/Mg ratios that relatively large differences in their initial ²⁶Al/²⁷Al ratios would, considering the experimental uncertainty², be unobservable. Thus, the determination of the source of the Mg and O anomalies will be difficult since we may not see the original distribution of ²⁶Al and excess ¹⁶O.

One other distinction remains between the Mg and O anomalies. While the ²⁶Al has to be associated with a nucleosynthetic event that occurred within a few million years of the Solar System's formation, the observed excess ¹⁶O does not. Since the (C) and (O) zones produce excess ¹⁶O, either 'old' presolar grains or 'last-event' supernova grains could have carried excess ¹⁶O into the presolar nebula. But, if grains cannot form in supernova ejecta, the only remaining way, in our scenario, to inject excess ¹⁶O is through last-event supernova gas.

The ²²Ne anomaly¹⁴ observed in CI chondrites may have been produced by 2.6 yr ²²Na-bearing presolar grains condensed in the (He) zone¹⁹. In the solar nebula these grains vaporise if $T_{\max} > 1,000$ K. But evidence²⁰ suggests that CI chondrites may have formed in nebular regions where $T_{\max} < 400$ K; if decay-produced ²²Ne can be retained in or on these grains at this temperature, incorporation of excess ²²Ne into CI chondrites may occur. But, irradiation¹⁷ in the early solar nebula could also produce excess ²²Ne with energy requirements far less stringent than those for the production of ²⁶Mg by irradiation.

What minerals could survive in the presolar nebula if the composition was solar except for a C/O ratio > 1 ? Enstatite chondrites apparently condensed in such a region²¹. Unfortunately, less is known about the mineralogical history of enstatite chondrites, so no limits on $T_{\max}$ can at present be imposed. Calculations¹¹ show that if $T_{\max} > 2,025, 1,930, 1,745, 1,458, 1,400$ and $1,390$ K, respectively, TiC, C, SiC, Fe₃C, AlN and CaS could survive: all of these phases can be condensed in at least two supernova zones (if C/O > 1 in the appropriate supernova zones). Thus, C, N, S and Fe, in addition to Mg, Si, Ca and Ti, isotopic measurements could potentially be key indicators of which supernova zones have been sampled. The C¹²/C¹³ ratio could prove very important in this respect, since this ratio should be less than its solar value if the (H) zone is responsible, while if presolar grains from the (He) and/or (C) zones are incorporated in enstatite chondrites this ratio should be greater than its solar value.

Before the discovery of the ²⁶Mg anomaly, it was thought that

the last nucleosynthetic event affecting the Solar System occurred about 10⁸ yr before its formation²², which is the interval between the production of the r-process radioactive parents (¹²⁹I, ²⁴⁴Pu) of Xe and the retention of daughter Xe in the meteorites. Assuming that the anomalous ²⁶Mg is the *in situ* decay product of ²⁶Al, the Mg timescale is about 10⁶ yr. There are at least two explanations for this discrepancy. First, ²⁶Al, ¹²⁹I and ²⁴⁴Pu may not have been produced in the same nucleosynthetic event, but in separate events 10⁸ yr apart. The latter event made ²⁶Al, but no r-process nuclei (or at least its r-process material did not get into the solar nebula). The incorporation of Al, I and Pu occurred about 10⁶ yr later. The second explanation is that these three nuclei were all made in the same (last) event, but somehow Xe was unable to be retained by meteorites until 10⁸ yr later, possibly because this region of the presolar nebula or the meteorite parent bodies remained too hot. A search for ²³⁵U anomalies due to the decay of ²⁴⁷Cm might resolve this problem²³. ²⁴⁷Cm (half life 1.54×10^7 yr), ¹²⁹I (1.7×10^7 yr) and ²⁴⁴Pu (8.3×10^7 yr) are all r-process nuclei. The U timescale's turning out to be of the order of the Xe timescale would be evidence in favour of the first possibility since U is a refractory element and does not diffuse out of meteoritic minerals as readily as gaseous Xe.

It seems, therefore, that a last-event supernova provides a consistent explanation of the observed isotopic anomalies. Condensation sequences for supernova ejecta imply that presolar grains may be a useful part of this picture.

We thank E. Anders, D. Arnett, R. Clayton, S. Falk and J. Truran for useful discussions. J.M.L. expresses appreciation for the hospitality of both the Institute of Astronomy, Cambridge, England, and NORDITA, Copenhagen, Denmark, where part of this research was done. This work was supported in part by the US NSF (J.M.L., D.N.S.), NASA (J.M.L., D.N.S., L.G.) and by the Alfred P. Sloan Foundation (L.G.).

Received 29 April; accepted 27 June 1977.

- ¹ Gray, C. M. & Compston, W. *Nature* **251**, 495 (1974).
- ² Lee, T. T., Papanastassiou, D. A. & Wasserburg, G. J. *Astrophys. J. Lett.* **211**, L107 (1977).
- ³ Schramm, D. N. *Astrophys. Space Sci.* **13**, 249 (1971); *Bull. Am. astr. Soc.*, Hawaii (1977).
- ⁴ Sabu, D. D. & Manuel, O. K. *Nature* **262**, 28 (1976); Cameron, A. G. W. & Truran, J. W. *Icarus* **30**, 447 (1977).
- ⁵ Woodward, P. R. *Astrophys. J.* **207**, 484 (1976).
- ⁶ Schramm, D. N. & Arnett, W. D. *Mercury* **4**, 16 (1975).
- ⁷ Howard, W. M., Arnett, W. D. & Clayton, D. D. *Astrophys. J.* **165**, 495 (1971).
- ⁸ Pardo, R. C., Couch, R. G. & Arnett, W. D. *Astrophys. J.* **191**, 711 (1974).
- ⁹ Woosley, S. E., Arnett, W. D. & Clayton, D. D. *Astrophys. J. Suppl.* **26**, 231 (1973).
- ¹⁰ Couch, R. G., Schmeidekamp, A. B. & Arnett, W. D. *Astrophys. J.* **190**, 95 (1974).
- ¹¹ Lattimer, J. M., Schramm, D. N. & Grossman, L. *Astrophys. J.* (1978).
- ¹² Cameron, A. G. W. in *Explosive Nucleosynthesis* (eds Schramm, D. N. & Arnett, W. D.) (University of Texas Press, Austin, 1973).
- ¹³ Clayton, R. N., Onuma, N., Grossman, L. & Mayeda, T. K. *Earth planet. Sci. Lett.* **34**, 209 (1977).
- ¹⁴ Blander, M. & Fuchs, L. H. *Geochim. cosmochim. Acta* **39**, 1605 (1975).
- ¹⁵ Grossman, L. & Ganapathy, R. *Geochim. cosmochim. Acta* **40**, 331 (1976).
- ¹⁶ Seitz, M. G. & Kushiro, I. *Science* **183**, 954 (1974).
- ¹⁷ Black, D. C. *Geochim. cosmochim. Acta* **136**, 377 (1972).
- ¹⁸ Eberhardt, P. *Earth planet. Sci. Lett.* **24**, 182 (1974).
- ¹⁹ Clayton, D. D. *Nature* **257**, 36 (1975).
- ²⁰ Grossman, L. *Scient. Am.* **232**, 30 (1975).
- ²¹ Larimer, J. W. *Geochim. cosmochim. Acta* **39**, 389 (1975).
- ²² Schramm, D. N. & Wasserburg, G. J. *Astrophys. J.* **162**, 57 (1970).
- ²³ Blake, J. B. & Schramm, D. N. *Nature phys. Sci.* **243**, 138 (1973).

Three-dimensional model of membrane-bound ribosomes obtained by electron microscopy

P. N. T. Unwin

MRC Laboratory of Molecular Biology, Hills Road, Cambridge, UK

A low-resolution three-dimensional map has been obtained from crystalline arrays of membrane-bound eukaryotic ribosomes. It shows both ribosomal subunits to be adjacent to the membrane surface, attached to it by a part protruding from the large subunit.

Most eukaryotic cells have two populations of actively synthesising ribosomes which are distinguishable morphologically according to whether they exist free in the cytoplasm or are bound to membranes, normally the endoplasmic reticulum. In general, the two populations synthesise different sets of proteins. One function of membrane-bound ribosomes, originally discovered from

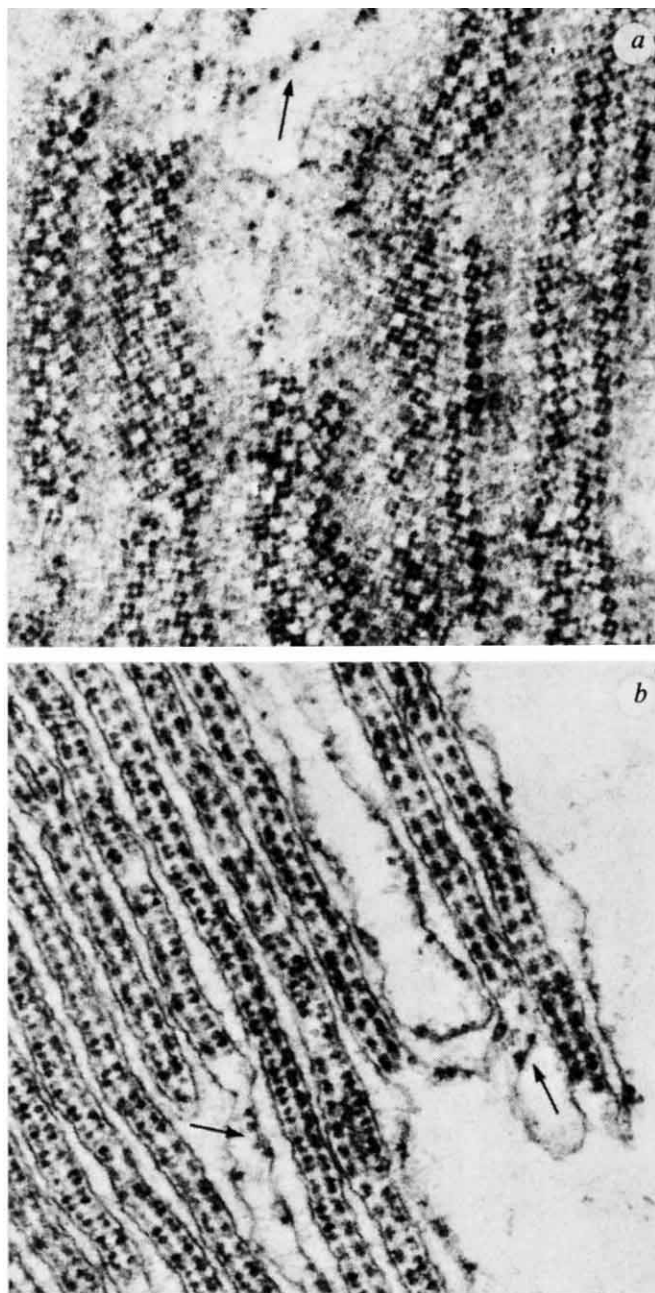


Fig. 1 Organisation of crystalline ribosomes and membranes as in the oocyte of *Lacerta sicula* during winter. *a*, Face-on view; *b*, edge-on view. Membrane-bound ribosomes which are separate from those in the crystals (arrows) are also evident ($\times 50,000$). To bring out details clearly, the ribosome-membrane complexes have been isolated from the cytoplasm and suspended in 100 mM KCl, 5 mM $MgCl_2$, 0.25 M sucrose, 50 mM triethanolamine-HCl, pH 7.6. All thin sections were prepared by fixing in glutaraldehyde, post-fixing in OsO_4 , embedding in Araldite, then staining after cutting with uranyl acetate and lead citrate.

experiments with secretory cells¹, is to synthesise proteins for export from the cell. These proteins are vectorially discharged through the membrane during synthesis².

Ribosomes are attached to the membranes by two types of interaction³; one is sensitive to ionic strength and the other to the antibiotic puromycin. The salt-sensitive interaction occurs directly between the large ribosomal subunit and the membrane, but its significance is not yet understood. The puromycin-sensitive interaction is indirect and is thought to be due to an anchoring effect by the membrane on the nascent polypeptide chain. It is therefore associated with proteins which are to be transferred across

the membrane. Recent findings indicate that the specificity for the interaction between the nascent polypeptide and the membrane resides in the amino-terminal sequence of the protein⁴⁻⁷.

It is generally assumed that the large ribosomal subunit lies between the small subunit and the membrane surface^{8,9} and that the protein being synthesised passes into and through the membrane through a protected region^{10,11} within the large subunit. But the electron microscopical and biochemical experiments on which these ideas are based do not provide definitive evidence, and the exact configuration of the ribosome with respect to the membrane is uncertain.

This study is based on a three-dimensional analysis of crystalline arrays of ribosomes which bind to membranes through a salt-sensitive linkage. The commonly assumed configuration is not confirmed. In the crystals, both subunits lie close to the membrane surface, attached to it by a part protruding from the large subunit.

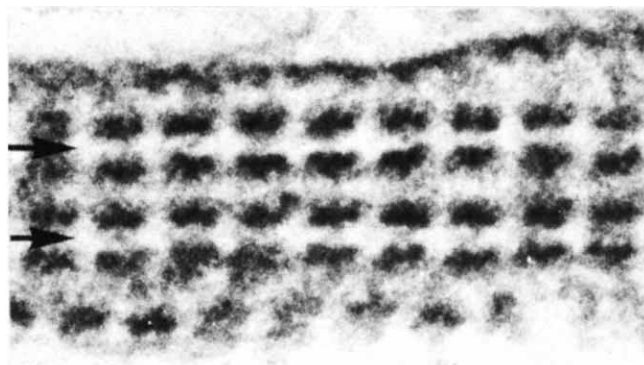
Lizard ribosome crystals

The crystalline sheets of ribosomes which develop in the oocytes of the lizard *Lacerta sicula* during its winter hibernation period¹², were used for this study. Each sheet is composed of two layers of ribosomes which are continuous with the cytoplasm and are bordered on either side by extended, flattened, vesicular membranes similar in appearance to the cisternae of the endoplasmic reticulum. In each layer of a sheet the ribosomes present the same surface to the membrane against which they are juxtaposed, and are arranged as tetramers in the space group P4 ($a=595 \text{ \AA}$) (ref. 13). The crystalline material is inactive *in vivo*, but seems to be competent in protein synthesis when isolated and tested in a cell-free system¹⁴.

Face-on and edge-on views of the sheets are shown in Fig. 1. A detailed description of the packing of the ribosomes seen there and of the crystallographic relations between the layers of a sheet has been given elsewhere¹³. Face-on views (Fig. 1*a*) looked at from an appropriate glancing angle, show either tetramers or single ribosomes to line up in rows. When, in the edge-on views (Fig. 1*b*), the tetramers or the single ribosomes superimpose, they give rise to characteristic patterns of dashed (tetramer) or dotted (single ribosome) lines.

The membranes are most obvious in the edge-on views. They are separated from the densely staining parts of

Fig. 2 Edge-on view of a six-layer stack of sheets formed by aggregation of three single sheets (each of two layers of ribosomes) in a medium of low ionic strength (50 mM KCl, 5 mM $MgCl_2$, 0.25 M sucrose, 50 mM triethanolamine-HCl, pH 7.6) containing 0.25% Triton. The zones from which the membranes have been extracted are indicated by the arrows. The other, darker, zones correspond to the region between the two layers of a sheet. Section $\sim 2000 \text{ \AA}$ thick ($\times 150,000$). Stacks of sheets formed artificially in this way have the same appearance as the 'three-dimensional crystals' of ribosomes which develop, in the absence of membranes, in live chick embryos when they are cooled¹⁵.



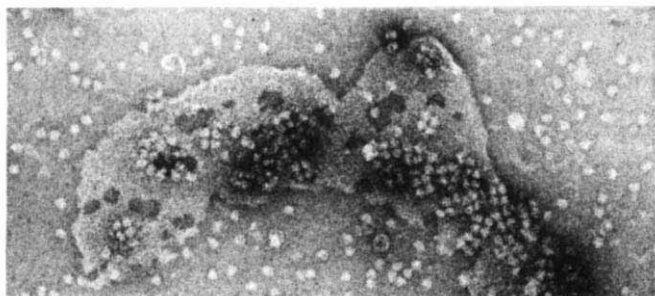


Fig. 3 Single ribosomes and tetramers attached to membrane fragments after high salt treatment; negatively stained in uranyl acetate ($\times 55,000$). The following steps were carried out at 4°C . The cytoplasm from about 50 selected previtellogenic oocytes were carefully opened out under a dissecting microscope into about $50\ \mu\text{l}$ of low salt medium (50 mM KCl, 5 mM MgCl_2 , 0.25 M sucrose, 50 mM triethanolamine-HCl, pH 7.6), stirred vigorously, then centrifuged at $\sim 300g$ for 10 min to produce a pellet consisting almost entirely of large aggregates of ribosome sheets and membranes. This pellet was resuspended in $50\ \mu\text{l}$ of high salt medium (500 mM KCl, 5 mM MgCl_2 , 0.25 M sucrose, 50 mM triethanolamine-HCl, pH 7.6), held for 15 min, and then centrifuged at higher speed to remove any large contaminating material. The supernatant, containing the freed ribosomes and membranes was divided into two equal portions, one of which was dialysed against the low salt medium for several hours. Both portions were subsequently examined together in the microscope. Only the dialysed portion contained membrane-bound ribosomes. The few mitochondrial membranes present in these solutions, and erythrocyte ghosts which had been added to them before dialysis, showed no signs of attachment.

the crystalline ribosomes by a narrow, but distinct, zone relatively free of stained material. A zone of the same width is also observed when the membranes have been partially dissolved, indicating that osmotic effects are not responsible for its appearance or its width. No gap corresponding to this zone is found, however, between ribosomes and membranes in the non-crystalline regions. These ribosomes always seem to be more closely attached, as do those on the endoplasmic reticulum.

Biochemical findings

The stain-depleted zone could be taken to indicate that the ribosome crystals are not attached to the membranes but are held apart, for example, by electrostatic forces. Other evidence favoured direct attachment, however: it was not possible to separate the components in detergent-free media of near physiological composition by normal mechanical means. In addition, the appearance of membrane-extracted stacks of sheets, which sometimes form in the presence of the detergent, Triton X-100 (see Fig. 2), seemed to imply that this zone contained some matter.

Two types of experiment gave more compelling evidence. In the first (Fig. 3) the KCl concentration was varied. It was found that the ribosome sheets, which seemed to be strongly attached to the membranes in solutions containing 5 mM MgCl_2 at KCl concentrations of about 100 mM or less, were all released without difficulty, and dissociated into single ribosomes and subunits, when it was raised to 500 mM. Furthermore, the released ribosomes were competent in attaching specifically to fragments of the same membrane when the KCl concentration was brought down again. As Fig. 3 shows, many, but not all, of the ribosomes which attached on lowering the KCl concentration tended to associate to form tetramers; the crystal bonding between tetramers was not re-established. In the second experiment (Fig. 4), ribosome sheets which had been isolated from the membranes by detergent treatment¹³, were incubated with vesicles of lizard liver endoplasmic reticulum from which

the bound ribosomes had been removed. A large proportion of the surfaces of all sheets then became covered by membranes, as would be expected if the ribosomes composing them were orientated so that their salt-sensitive attachment sites were exposed and available for interaction. A strong association is suggested by the observations that most of the vesicles are drawn out in single membrane layers and that in places where they are only on one side they cause the crystal to bend.

I conclude that the ribosomes are directly attached to the membranes through a salt-sensitive linkage which may well be the same linkage that binds them to the endoplasmic reticulum.

Analysis of isolated sheets

To investigate the three-dimensional structure of the ribosomes in the sheets they were isolated from the membranes as in ref. 13, placed on electron microscope grids and negatively stained with uranyl acetate. Micrographs were then taken of them tilted at angles between 0° and 76° to the incident 100-kV electron beam.

Fourier methods^{17,18} were used to analyse and combine the data from the micrographs. The Fourier transform of one layer of ribosomes is a two-dimensional lattice of lines along which the phases and amplitudes vary continuously. The Fourier transform of an isolated sheet therefore consists of two such lattices, one of which is

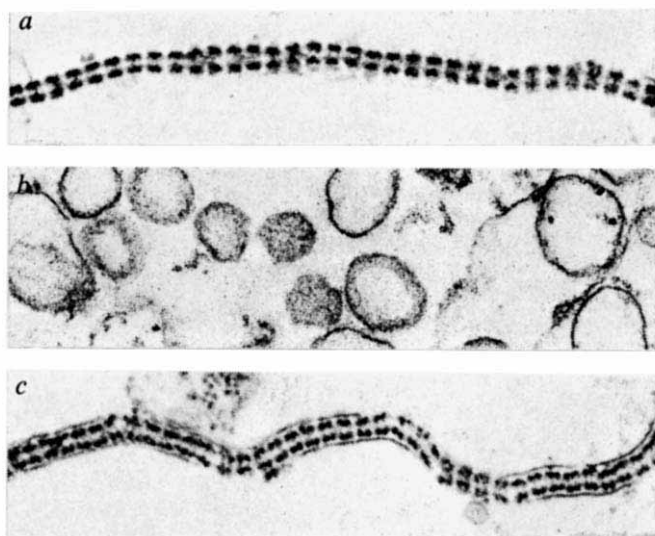


Fig. 4 Isolated crystalline sheets of ribosomes, (a), were incubated with vesicles of endoplasmic reticulum (b), giving the result, (c) ($\times 40,000$). Vesicles of endoplasmic reticulum were prepared at 4°C by a procedure similar to that given in ref. 16. two lizard livers were homogenised in about twice the volume of a medium containing 0.25 M sucrose, 50 mM triethanolamine-HCl, pH 7.6, and centrifuged twice at 15,000 r.p.m. ($\sim 20,000g$) in a M.S.E. 3X6.5 titanium swing-out rotor, discarding the pellet and scum at the top on each occasion. The supernatant was then layered on to a discontinuous gradient of 1.5 M and 2.0 M sucrose in 50 mM KCl, 5 mM MgCl_2 , 50 mM triethanolamine-HCl, pH 7.6, and spun at 42,000 r.p.m. ($\sim 150,000g$) for 20 h, using the same rotor. The slightly opalescent 'rough microscope fraction' was withdrawn from the region near the 1.5 M/2 M sucrose interface and mixed with an equal volume of a high salt medium containing puromycin so that the final concentration was 500 mM KCl, 5 mM MgCl_2 , 0.75 M sucrose, 50 mM triethanolamine-HCl, 1 mM puromycin, pH 7.6. This mixture was incubated at 22°C for 30 min to release the ribosomes and then passed through the same sucrose gradient as previously. The ribosome-stripped vesicle fraction was removed from the second centrifugation at a concentration of ~ 4 mg of membrane protein per ml. The vesicle preparation was mixed with an equal volume of isolated ribosome sheets in 50 mM KCl, 5 mM MgCl_2 , 0.25 M sucrose, 50 mM triethanolamine-HCl, pH 7.6, prepared as before¹³, and incubated for 6 h at 0°C before fixation, embedding and sectioning.

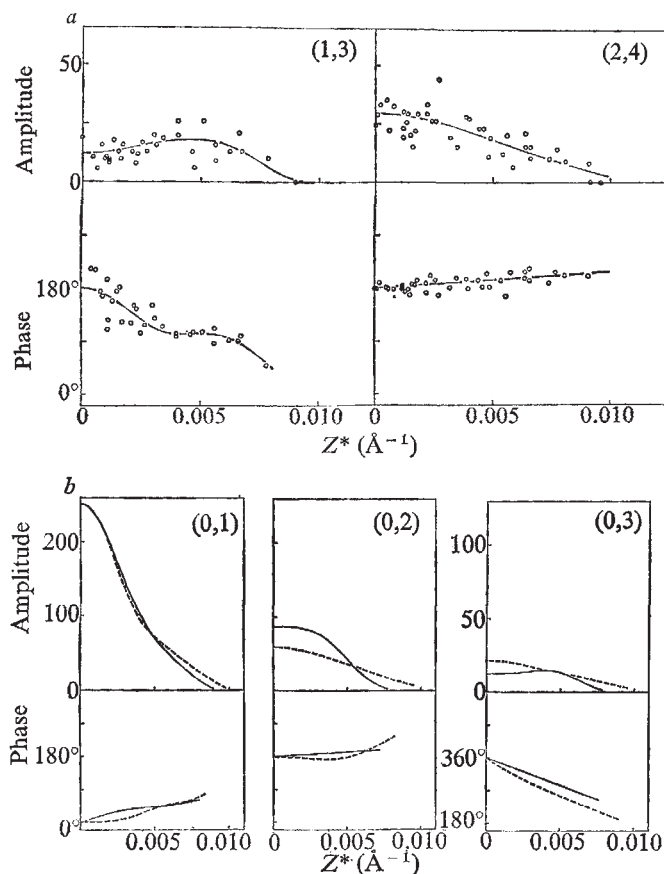


Fig. 5 *a*, Values of calculated amplitudes and phases along two typical lattice lines, plotted as a function of distance (Z^*) from their centres. In this space group the Friedel relationship gives the corresponding values for negative Z^* . Continuous curves drawn through the points and sampled every $0.002 \text{ \AA}^{-1}$ give the data on which the three-dimensional map is based. The average phase error, based on comparison of single phase measurements against all others within a small range of Z^* , is less than 20° . Micrographs were taken with a goniometer stage, using bent support grids to achieve the highest tilt angles. They were densitometered and analysed as in ref. 13. The information was combined as in ref. 18. No corrections for effects of defocus were needed at this resolution. *b*, Amplitudes and phases calculated for the $(0,k)$ lattice lines from the tilting experiments (full line) and from edge-on views of superimposed tetramers in $\sim 2,000\text{-\AA}$ thick sections (broken line). The data obtained from the edge-on views have been adjusted to give the best fit with the data from the tilting experiments, the difference between positive and negative staining being accounted for by a 180° phase change. The amplitude scale factor and phase origin adjustment needed to fit the two sets of data were used to correct the variation along the $(0,0)$ lattice line, which the edge-on view gives directly.

upside-down and rotated by a specific amount with respect to the other, depending on the crystallographic relationship between the two layers. Hence, each micrograph gives two sections through the three-dimensional Fourier transform of one layer.

Sixteen micrographs (32 different views of one layer) were needed to give continuous curves of phase and amplitude along all of the 25 crystallographically independent lattice lines, except for the line through the origin, out to a resolution of $90 \text{ \AA}$. Two such curves are shown in Fig. 5*a*. Several micrographs from a given tilt series were suitable for data collection since radiation damage was not a limiting factor.

Optical diffraction patterns from micrographs of negatively stained and (sectioned) positively stained isolated sheets were similar, indicating that at this low resolution, except for the reversal in contrast, positively stained and negatively stained sheets would provide roughly

equivalent maps of the structure. The approximate form of the lattice line through the origin could therefore be determined from sectioned material by calculating the Fourier transform of an edge-on view of one layer. The correct scale of the amplitudes and the origin adjustment for the phases along this lattice line were then found by comparing data, as in Fig. 5*b*.

By sampling the continuous variation of the complete transform at suitably fine intervals ($0.002 \text{ \AA}^{-1}$) along the lattice lines, a three-dimensional map of the ribosomes in one layer was calculated.

Three-dimensional map

Figure 6 shows a tetrameric unit from the three-dimensional map, viewed so that the juxtaposed membrane, if it were present, would lie underneath, and also a single ribosome from this unit, viewed parallel to the plane of the array. The contours map out the negative stain-excluding regions only. Therefore they are insensitive to differences of composition within the ribosome and do not show up areas of RNA or protein into which the negative stain may have penetrated.

Particularly noticeable in Fig. 6*a* is a region within the ribosomes which looks like a hole, and so probably represents that part where the large and small ribosomal subunits are most widely separated. The shape and dimensions of the matter surrounding this region resemble features observed in micrographs of isolated eukaryotic ribosomes and of their isolated subunits^{19,20}. A detailed comparison, and arguments based on molecular weight estimates²¹, indicate that the two subunits lie side by side, and suggest that the ribosome can be partitioned as shown, with the large subunit closest to the centre of the tetramer. The line dividing the subunits seems to follow the path in which the inner contours come closest together and connect the 'hole' to the outside, but its exact position cannot be determined from this low resolution map.

Figure 6*b*, the view of a single ribosome along the direction of this path, emphasises another feature of the other map—a narrow protrusion on the large subunit

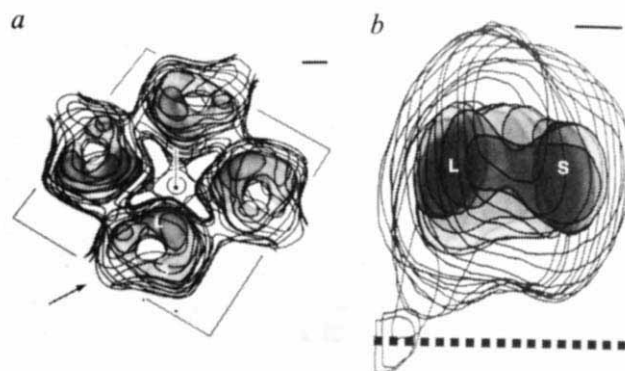


Fig. 6 Views of *a*, a tetramer and *b*, a single ribosome emphasising the dominant features of the three-dimensional map. The negative contours, representing the stain excluding parts, have been drawn at two levels (-2 and -6 in a scale 0 to -10) and the region within the inner contours has been shaded. The tetramer is orientated so that the juxtaposed membrane would lie underneath (right-handed configuration¹³); it is upside-down in comparison with the usual orientation on microscope grids of isolated tetramers and tetramers in p4 arrays²², where the ribosomes present 'left featured frontal' views^{19,20}; thus the small subunit (S) is on the anticlockwise side, and the large subunit (L) on the clockwise side, of the path (arrowed) connecting the 'hole' to the outside. The single ribosome is viewed from the direction indicated by the arrow; the feature protruding from it was estimated, independently of the map, to extend as far ($\pm 15 \text{ \AA}$) as the dotted line. Scale bars represent $50 \text{ \AA}$.

extending towards the central fourfold axis. From the configuration of the group of four of them, it seems that the ribosomes of a tetramer touch in the region close to the central fourfold axis, as well as at a higher radius. These protrusions are unlikely to be something peculiar to ribosomes which crystallise, since the tetramers can be formed *in vitro* from ribosomal subunits released from polysomes²². Nor are they likely to be membrane proteins, since another explanation would then be required for the identical packing of ribosome tetramers in the non-membrane associated stacks of sheets found in chick embryos when they are cooled¹⁵. They are therefore most probably normal components of the large subunits.

Membrane attachment

Edge-on views of stacks of sheets (Fig. 2) were used to derive, independently of the three-dimensional map, the distance reached by the protrusions in the direction of the membrane. In the membrane-extracted zones of these stacks the protrusions show up as slightly denser areas between the dashed lines formed by the tetramers, and presumably extend halfway into the zones. The half-way distance was estimated from projection maps calculated from Fourier transforms of these views, using the data on which the three-dimensional map is based to obtain the correct scale and to define the centre of a layer. The result of the estimate, the furthestmost distance reached by the protrusions in the direction of the membrane, is indicated by the dotted line drawn in Fig. 6b. The position of this line is consistent with the details in the map.

The line in Fig. 6b could also represent the surface of the membrane with which the crystalline ribosomes are associated *in vivo*, since the open space between the protrusions would then give a zone of the same width as seen in Fig. 1b. No additional material is required to span the space. Therefore the protrusions must be the links by which the crystalline ribosomes establish contact with sites on the membrane surface.

It seems unnecessary to draw a distinction in mode of attachment between the crystalline and non-crystalline membrane-bound ribosomes. The closer association with

the membrane of the latter could be due to some freedom of movement of the ribosomes about their attachment site when they are no longer constrained by the crystal bonding.

It is possible that membrane-bound ribosomes are attached by the same mode—some distance from the body of the ribosome—during the initial stages of protein synthesis. A wide separation between the site where the large subunit binds and the emerging polypeptide should allow the polypeptide freedom of motion relative to the highly fluid^{23,24} endoplasmic reticulum, without greatly disturbing its proximity or orientational disposition. Thus, the main function of this type of attachment could be to give the molecules in the membrane and the protein being synthesised a good chance to interact.

I thank Nichol Thompson for cutting the sections, Dr L. Amos for some computer programs and Drs C. Taddei, R. Henderson and M. Bretscher for helpful discussions. The Philips Application Laboratory Cambridge, kindly made available the goniometer version of their EM400 electron microscope for the tilting experiments.

Received 13 May; accepted 8 July 1977.

- 1 Siekevitz, P. & Palade, G. E. *J. biophys. biochem. Cytol.* **7**, 619–644 (1960).
- 2 Redman, C. M., Siekevitz, P. & Palade, G. E. *J. biol. Chem.* **241**, 1150–1158 (1966).
- 3 Adelman, M. R., Sabatini, D. D. & Blobel, G. *J. Cell Biol.* **56**, 206–229 (1973).
- 4 Milstein, C., Brownlee, G. G., Harrison, T. M. & Mathews, M. B. *Nature new Biol.* **239**, 117–120 (1972).
- 5 Blobel, G. & Dobberstein, B. *J. Cell Biol.* **67**, 835–851 (1975).
- 6 De Villiers-Thiery, A., Kindt, T., Scheele, G. & Blobel, G. *Proc. natn. Acad. Sci. U.S.A.* **72**, 5016–5020 (1975).
- 7 Wirth, D. F., Katz, F., Small, B. & Lodish, H. F. *Cell* **10**, 253–263 (1977).
- 8 Sabatini, D. D., Tashiro, Y. & Palade, G. E. *J. molec. Biol.* **19**, 503–524 (1966).
- 9 Redman, C. M. & Sabatini, D. D. *Proc. natn. Acad. Sci. U.S.A.* **56**, 608–615 (1966).
- 10 Malkin, L. I. & Rich, A. *J. molec. Biol.* **26**, 329–346 (1967).
- 11 Blobel, G. & Sabatini, D. D. *J. Cell Biol.* **45**, 130–145 (1970).
- 12 Taddei, C. *Expl Cell Res.* **70**, 285–292 (1972).
- 13 Unwin, P. N. T. & Taddei, C. *J. molec. Biol.* **114**, 491–506 (1977).
- 14 Taddei, C., Gambino, R., Metafora, S. & Monroy, A. *Expl Cell Res.* **78**, 159–169 (1973).
- 15 Byers, B. *J. molec. Biol.* **26**, 155–167 (1967).
- 16 Adelman, M. R., Blobel, G. & Sabatini, D. D. *J. Cell Biol.* **56**, 191–205 (1973).
- 17 DeRosier, D. J. & Klug, A. *Nature* **217**, 130–134 (1968).
- 18 Henderson, R. & Unwin, P. N. T. *Nature* **257**, 28–32 (1975).
- 19 Nonomura, Y., Blobel, G. & Sabatini, D. D. *J. molec. Biol.* **60**, 303–323 (1971).
- 20 Lake, J. A., Sabatini, D. D. & Nonomura, Y. in *Ribosomes* (eds Nomura, M., Tissières, A. & Lengyel, P.) 543–557 (Cold Spring Harbor Laboratory Press, New York, 1974).
- 21 Wool, I. G. & Stöffler, G. in *Ribosomes* (eds Nomura, M., Tissières, A. & Lengyel, P.) 417–460 (Cold Spring Harbor Laboratory Press, New York, 1974).
- 22 Byers, B. *Proc. natn. Acad. Sci. U.S.A.* **68**, 440–444 (1971).
- 23 Rogers, M. J. & Strittmatter, P. *J. biol. Chem.* **249**, 895–900 (1974).
- 24 Ojakian, G. K., Kreibich, G. & Sabatini, D. D. *J. Cell Biol.* **72**, 530–551 (1977).

In vitro synthesis of infectious DNA of murine leukaemia virus

Ellen Rothenberg, David Smotkin,
David Baltimore & Robert A. Weinberg

Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

DNA synthesised in vitro by purified virions of murine leukaemia virus is infectious. Neither RNA nor protein is required for infectivity. Transfection with reverse transcriptase product shows a single-hit dose response and results in the production of complete, infectious virus.

THE discovery of reverse transcriptase in virions of retroviruses^{1,2} provided a system for studying the viral DNA synthesis which was known to be essential early in infection^{3,4}. Until recently, however, most groups studying reverse transcription *in vitro* found the DNA products to be small relative to the size of the RNA templates. Thus it was not known whether virions could make a complete copy of the

viral genome without the intervention of cellular factors.

Recent studies have improved reaction conditions so as to allow detergent-disrupted virions to synthesise DNA molecules up to 8,000–9,000 nucleotides long, the length of the viral genome RNA which serves as template^{5–7}. It has been possible to synthesise very long DNA transcripts in the 'endogenous reaction' without sacrificing the specificity of initiation: the longest products made by virions of Moloney murine leukaemia virus (M-MuLV) apparently share the unique initiation site and tRNA^{Pro} priming mechanism used by the shorter transcripts^{8,9}. Since the longest transcripts also migrate as a discrete species close to genome length in Agarose gel electrophoresis, it seemed possible that they could be biologically active. We report here that infectious DNA can be synthesised *in vitro* by purified virions of M-MuLV.

Conditions for synthesis of infectious molecules

The conditions used for DNA synthesis were essentially the same as described previously⁶ with a few modifications. Actinomycin D was omitted except where indicated below. A higher yield of the longest DNA molecules was obtained when the ionic strength was reduced to a minimum by omission of NaCl from the reaction mixture and when the incubation was carried out at temperatures between 39 and 41 °C. In most of the DNA preparations used for these studies, each deoxyribonucleoside triphosphate (dNTP) was present at 2 mM. In early studies, DNA was synthesised with each dNTP at 5 mM, but a higher absolute yield of the longest transcripts was obtained with the lower substrate concentration. The lower yield at high substrate concentration was presumably due to the inhibitory effects of the 40–60-mM Na⁺ ions contributed by 20 mM nucleotides. The concentration of magnesium acetate used in these studies was 6.6–7.2 mM, just lower than the total dNTP concentration of 8 mM. Synthesis of long DNA molecules proceeded for 12–20 h, and the incubation could be continued for at least 40 h without detectable degradation of the product DNA.

The products of reverse transcriptase reactions were assayed for infectivity by the Ca₃(PO₄)₂ coprecipitation method of Graham and van der Eb^{10,11} as previously described^{12,13}. The total nucleic acid was extracted from an incubation mixture with sodium dodecyl sulphate, phenol, and chloroform, passed over a column of Sephadex G-50, and ethanol precipitated. In most experiments, virion RNA was not removed before transfection. After exposure to the nucleic acid, NIH/3T3 cells derived from NIH/Swiss mice were scored for successful infection using the syncytium-forming assay with an XC cell overlay as previously described^{12,14}.

Unincubated virions and virions incubated in suboptimal conditions for DNA synthesis were consistently devoid of infectious nucleic acid. After 3–4 h of incubation in the conditions described above, however, infectivity could be detected in the reaction product and increased thereafter for at least 12 h (Fig. 1).

Transfection yields complete virus

To determine whether the transfected cells forming syncytia with XC cells were producing complete virions, the culture fluids of NIH/3T3 cells transfected with endogenous reaction product were assayed for infectious virus. Titres of 10²–10³ PFU per 10 ml culture fluid were found within 3 d of transfection in every culture yielding even one or two infective centres by primary XC assay. After 15 d culture, the supernatants of transfected cells yielded titres of over 10⁶ PFU ml⁻¹. Mock-transfected cells always remained virus free. Therefore transfection with reverse transcriptase products resulted in the production of infectious virus.

The fact that virus spread efficiently through the transfected culture suggested that it was not an endogenous virus, since the only reported endogenous virus of NIH/Swiss mice is xenotropic, that is, incapable of infecting murine cells^{3,5}. To confirm the origin of the transfection-derived virus, it was tested for the distinctive host range of the parent M-MuLV. M-MuLV grows equally well on NIH/3T3 (N-type) and BALB/3T3 (B-type) cells, but no known endogenous virus of mice can grow on both. When the transfection-derived virus and M-MuLV were titred on NIH/3T3 and BALB/3T3 cells, the ratio of the titre on N cells to the titre on B cells was 2.9 for M-MuLV and 2.4 for the transfection-derived virus. As controls, an N-tropic virus stock was shown to have a 100-fold preference for the N-type cells, while a B-tropic virus stock had a 10,000-fold preference for B-type cells. Thus the transfection-derived virus displayed the host range of the M-MuLV used in the reverse transcriptase reaction, and not of any known endogenous virus. Unless every infective centre resulted from recombination of M-MuLV fragments specifying host range with an endogenous viral genome, the product of incubated virions contained all the essential genetic information of M-MuLV.

Structure of infectious molecules

The biochemical nature of the infectious molecules was studied by subjecting them to various treatments (Table 1). The infectivity was unaffected by protease, and resistant to RNase at low ionic

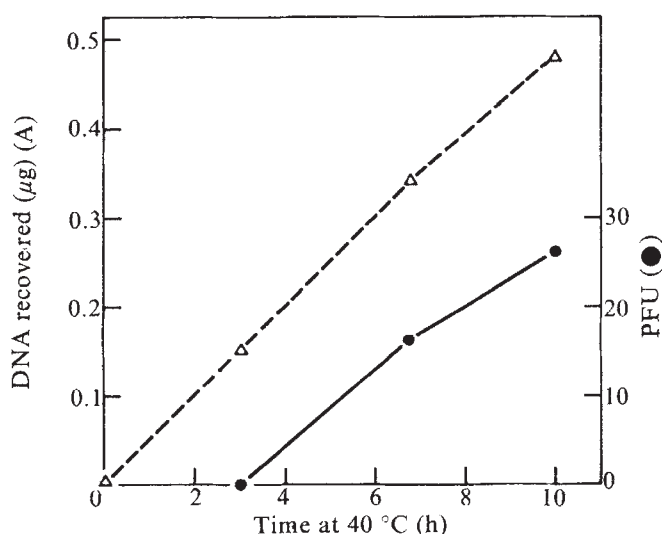


Fig. 1 Synthesis of infectious DNA *in vitro*. Virions were incubated in an endogenous reaction mixture containing 320 c.p.m. pmol⁻¹ ³H-dCTP. At the indicated times, the nucleic acid was extracted from a sample, freed of unincorporated substrate, and used to transfect two plates of 1 × 10⁶ NIH/3T3 cells. The amount of DNA in each sample was determined by monitoring the radioactivity immediately before the addition of CaCl₂. Each point shows the total number of XC plaques obtained with a DNA sample corresponding to about 90 μg of virus protein. Δ, DNA recovered; ●, PFU.

strength, a treatment which will solubilise even RNA hybridised to DNA. Deoxyribonuclease completely abolished the infectivity. Therefore the infectivity must depend on DNA but not on a DNA-protein or DNA-RNA complex. The infectivity was not affected by heating to 68 °C in low salt to disrupt aggregates or by denaturation at 37 °C in 0.1 M NaOH. Complete resistance of the infectivity to alkaline denaturation was observed in the four independent DNA preparations tested. The implications of this result for the structure of the infectious molecules are discussed below.

Since the specific infectivity of the total endogenous reaction product was at least three orders of magnitude lower than that of purified proviral DNA (ref. 12) relatively high concentrations of reverse transcriptase product had to be applied to the cells. Thus it was conceivable that much or most of the infectivity was due to complementation among different partial transcripts. Two different DNA preparations of similar specific infectivity were used to study the dose-response of the transfection. In both cases (Fig. 2)

Table 1 Infectivity of reverse transcriptase product after different treatments

Treatment	XC plaques	DNA (μg)	PFU μg ⁻¹
None	54	0.32	167
68 °C	43	0.31	137
0.1 M NaOH	45	0.29	154
Proteinase K	45	0.25	178
RNase	15	0.14	105
DNase	0	<0.02	—

Virus at 1.6 mg ml⁻¹ was incubated in an endogenous reaction containing 15 mM Mg acetate, 60 mM NaCl, and each dNTP at 5 mM. Incubation was for 7 h at 37 °C. After deproteinisation and gel filtration, six portions were ethanol precipitated. The control was used for transfection in 1 ml of HEPES-buffered saline in standard conditions. One sample was dissolved in 50 μl of 2 mM Tris-HCl, pH 8, 2.2 mM EDTA, and incubated for 5 min at 68 °C before addition of HEPES-buffered saline. One sample was dissolved in 20 μl 0.1 M NaOH and incubated for 15 min at 37 °C, then chilled, diluted with HEPES-buffered saline, and adjusted to pH 7.05 with acetic acid. RNase digestion was for 30 min at 37 °C with 50 μg ml⁻³ pancreatic RNase in 2 mM Tris-HCl pH 7.5, 1 mM EDTA. DNase treatment was for 30 min at 37 °C with 20 μg ml⁻¹ DNase in 2 mM Tris-HCl, pH 7.5, 10 mM MgCl₂. Nuclease-treated samples anti control were subsequently treated with 1 mg ml⁻¹ proteinase K for 10 min at 37 °C and extracted twice with chloroform iso-myl alcohol. They were ethanol precipitated again before transfection.

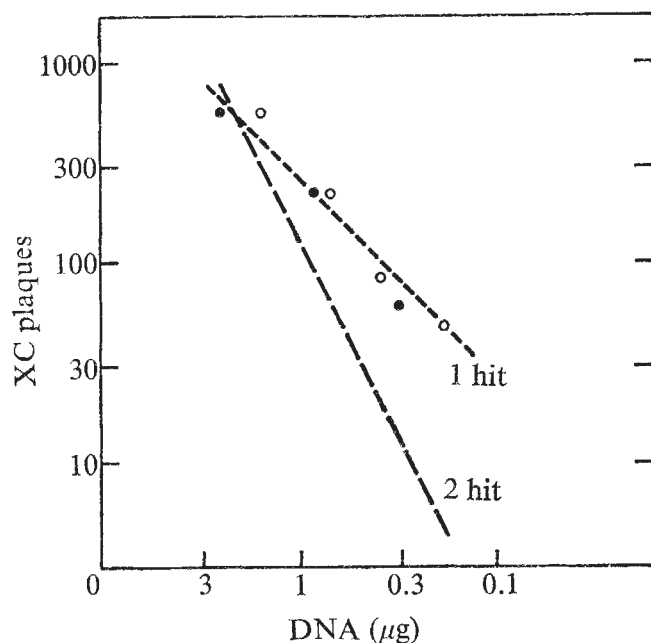


Fig. 2 Dose-response of transfection with endogenous reaction product. ^3H -DNA with a specific activity of $42,400 \text{ c.p.m. } \mu\text{g}^{-1}$ was synthesised from two different harvests of M-MuLV, as described in the legend to Fig. 1. Incubation was for 13.7 h at 40°C . One DNA preparation ($\bullet$) was subjected to serial threefold dilutions, and the other ($\circ$) was used in twofold dilutions. Both sets of samples were then ethanol precipitated and used to transfect duplicate plates of cells. The concentration of DNA in each dilution was ascertained by measuring the radioactivity in an aliquot. The broken lines represent the theoretical curves expected for one-hit and two-hit processes giving 500 plaques per $2 \mu\text{g}$ DNA.

the titration showed single-hit kinetics. On a double-logarithmic plot, the slopes of the lines giving the best least-squares fit through each set of points were 1.09 ± 0.10 and 1.22 ± 0.10 . Thus it is most likely that all the genetic information of M-MuLV was carried in individual molecules or molecular complexes resulting from reverse transcription.

Effects of actinomycin D

The alkali resistance of the infectivity, noted in Table 1, suggested that duplex structure might not be required. If single strands of DNA were infectious, then actinomycin D, which selectively blocks synthesis of the second (positive) strand, might be expected to allow infectious molecules to be made. Pairs of DNA samples synthesised in parallel with and without the inhibitor were therefore tested for infectivity (Table 2). In each case, the sample made in the presence of the drug lacked detectable infectivity, whereas the parallel sample made in the absence of the drug was highly infectious. The difference was not due to persistence of small amounts of actinomycin D through the phenol extraction, which might inhibit transfection directly, because a sample made in the absence of the drug remained infectious after addition of $100 \mu\text{g ml}^{-1}$ actinomycin D followed by phenol extraction. Thus actinomycin D prevented the synthesis of infectious molecules.

DNA preparations made with or without actinomycin D were compared in an attempt to explain the difference in biological activity. After 12 h of synthesis, the DNA made in the absence of the drug consistently banded in isopycnic gradients of NaI as almost fully double stranded¹⁶. Actinomycin D, at $100 \mu\text{g ml}^{-1}$, completely suppressed the formation of double-stranded molecules, allowing only the synthesis of negative-strand DNA (not shown). This result is consistent with previous reports^{17,18}. Another difference was found, however, when the ^3H -labelled products of reactions with and without the drug were fractionated by electrophoresis through alkaline Agarose gels, and the DNA displayed either by staining with ethidium bromide (Fig. 3a) or by

fluorography of incorporated tritium (Fig. 3b). In completely denaturing conditions¹⁹, products of both reaction mixtures migrated as an array of species with molecular weights of up to 2.5×10^6 – 3.0×10^6 . The majority of the additional DNA made in the absence of actinomycin D was smaller than about 2×10^6 daltons. This size range is consistent with the size of the majority of positive-strand DNA found *in vivo*²⁰. As previously reported⁶, the longest molecules in each sample formed a discrete band (arrows in Fig. 3b). In the absence of the inhibitor, however, the most extensive transcripts were about 0.2×10^6 MW (600 nucleotides) larger than the largest species made in the presence of the drug (compare lanes 1 and 2, Fig. 3). Both species were capable of hybridising to ^{125}I -labelled virion RNA, and so both represented the first (negative) strand of DNA (F. Yoshimura & E. Rothenberg, unpublished). Thus actinomycin D apparently blocked the addition of about 600 nucleotides to the longest negative-strand polymerase products. The biological deficiency of DNA made in the presence of the drug could therefore be attributed to either of two effects: the absence of positive-strand DNA, or the incompleteness of the negative-strand transcripts.

Sedimentation of infectious molecules

To relate the infectivity more closely to the structure of the DNA, the total endogenous reaction product was fractionated by sedimentation through neutral and alkaline sucrose gradients and the fractions were assayed for infectivity. In the neutral sucrose gradient, the pattern of infectivity was comparable with that reported for the linear cytoplasmic viral DNA extracted from infected cells^{12,21}. A peak of infectivity was observed sedimenting at 18–20S, with no infectivity in material sedimenting more slowly than linear SV40 DNA (Fig. 4a). This is consistent with double-stranded linear molecules of full genome size, 8,000–9,000 nucleotides long. In contrast to viral DNA found *in vivo*¹², the endogenous reaction product contained a relatively high proportion of infectivity sedimenting rapidly from 20S to about 60S. These molecules might be DNA concatemers or DNA still associated with remnants of the 70S RNA complex. Since DNA synthesised in these conditions was almost completely double stranded, it was unlikely that any appreciable fraction of the rapidly-sedimenting infectious material might be single-stranded DNA of genome length.

If denatured endogenous reaction products were infectious, full infectivity should be retained by a sample fractionated on an alkaline sucrose gradient. This was not the case: in three separate attempts, only about 10% of the infectivity could be recovered from these gradients (Fig. 4b). Virtually no DNA was lost during

Table 2 Effect of actinomycin D on synthesis of infectious DNA

Sample synthesised with	DNA (μg)	PFU	PFU μg^{-1}
1 No actinomycin D	1.9	172	92
100 $\mu\text{g ml}^{-1}$ actinomycin D	0.57	0	<1.8
2 No actinomycin D	0.90	92	102
100 $\mu\text{g ml}^{-1}$ actinomycin D	0.35	0	<2.9
3 No actinomycin D	0.78	309	396
100 $\mu\text{g ml}^{-1}$ actinomycin D	0.28	0	<3.8
4 No actinomycin D	1.5	558	365
No actinomycin D; 100 $\mu\text{g ml}^{-1}$ actinomycin D added after incubation	1.5	338	224

Pairs of DNA samples labelled with ^3H -dCTP were synthesised in parallel with or without $100 \mu\text{g ml}^{-1}$ actinomycin D. Different virus preparations were used for different experiments, in incubations of 19 h (experiment 1) or 16 h (experiments 2 and 3) at 40°C . The DNA sample used in experiment 4 was one of the samples described in the legend to Fig. 2. Each sample was used to transfect duplicate plates of NIH/3T3 cells, monitoring the input of DNA by radioactivity. In experiment 4, actinomycin D was added to a sample of the purified DNA preparation to a final concentration of $100 \mu\text{g ml}^{-1}$; the sample then underwent two extractions with phenol and one with chloroform before ethanol precipitation. The control sample in experiment 4 was precipitated directly without extraction.

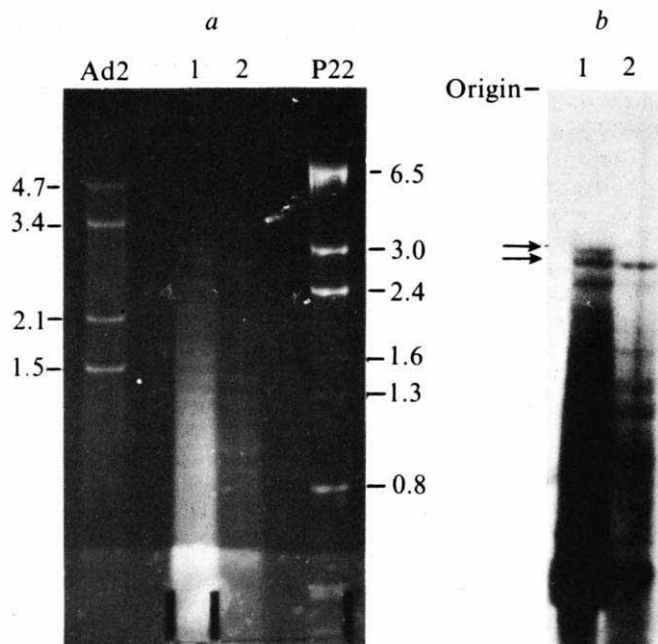


Fig. 3 Effect of actinomycin D on size of reverse transcriptase products. ^3H -labelled DNA samples synthesised in the absence and presence of actinomycin D (a gift of Merck, Sharp and Dohme), as described in the legend to Table 2 (experiment 3), were analysed by electrophoresis on a slab gel of 1% Agarose in the denaturing conditions described by McDonnell *et al.*¹⁹. The gel was cast in 30 mM NaCl, 2 mM EDTA on a plug of 2% Agarose in the same buffer; the samples were applied in 2 mM Tris-HCl, pH 7.5 1 mM EDTA for 13 h at 25 V. The gel was stained with $0.5 \mu\text{g ml}^{-1}$ ethidium bromide²² and photographed during ultraviolet excitation (a). The same gel was treated for fluorography²³ and exposed to film for 6 d at -70°C . Lane 1: DNA synthesised in the absence of the drug. Lane 2: DNA synthesised in the presence of $100 \mu\text{g ml}^{-1}$ actinomycin D. The single-strand molecular weights ($\times 10^{-6}$) of the marker DNAs, adenovirus type 2 cleaved with *Bam*HI endonuclease and phage P22 cleaved with *Eco*RI endonuclease, were as given by H. Delius, R. Greene, and C. Mulder (unpublished), and E. N. Jackson, H. I. Miller, and M. L. Adams (submitted for publication), respectively. Two additional single-strand fragments in the P22 digest were previously reported⁶.

the fractionation, and control samples not submitted to fractionation retained their infectivity. The longest DNA molecules made *in vitro* had previously been shown to resediment on alkaline sucrose gradients without evidence of degradation⁶ so that it seemed unlikely that breakage was responsible for the loss of infectivity. Furthermore, the remaining biological activity sedimented not as full-length transcripts, which should be in fractions 7 and 8 (Fig. 4b), but as molecules of only 4,000–5,000 nucleotides (fractions 9–10), between the full-length single strands and the bulk of the DNA (fractions 11–12, Fig. 4b). Similarly, when a sample identical to that shown in Fig. 4a was denatured in alkali before fractionation on a neutral sucrose gradient, once again only 10% of the infectivity was recovered. As observed with the alkaline sucrose gradient fractionation, the residual infectivity in these neutral sucrose gradients was found in material sedimenting more rapidly than the bulk of the DNA but more slowly than full-length single strands (not shown).

These results present two striking paradoxes: first, that infectious molecules should sediment in alkali similarly to DNA of only half genome length, and second, that DNA which quantitatively retained its infectivity through complete alkaline denaturation should lose biological activity if fractionated by size before transfection.

Discussion

The drastic loss of infectivity in these experiments suggested that size fractionation of the denatured DNA could be separating components which were jointly required for biological activity. The infectivity recovered from the alkaline sucrose gradient could

then result from reassociation of these components. The nature of these cooperating components is suggested by the observation, noted above, that the genome-length transcripts seem to represent the negative strand, whereas the positive strand molecules seem to be distinctly shorter. Size fractionation of the denatured reverse transcriptase products would thus result in the separation of all positive strand DNA from the only molecules which were genetically complete. We therefore believe it most likely that for a molecule to be infectious, the cooperation of a full-length negative strand and some shorter positive-strand DNA is required. On this interpretation, the small peak of infectivity in Fig. 4b would represent the limited overlap of the peaks of full-length negative strands and incomplete positive strands, and the quantitative resistance of the infectivity to simple alkaline denaturation (Table 1) would be an artefact of rapid reassociation. The positive-strand

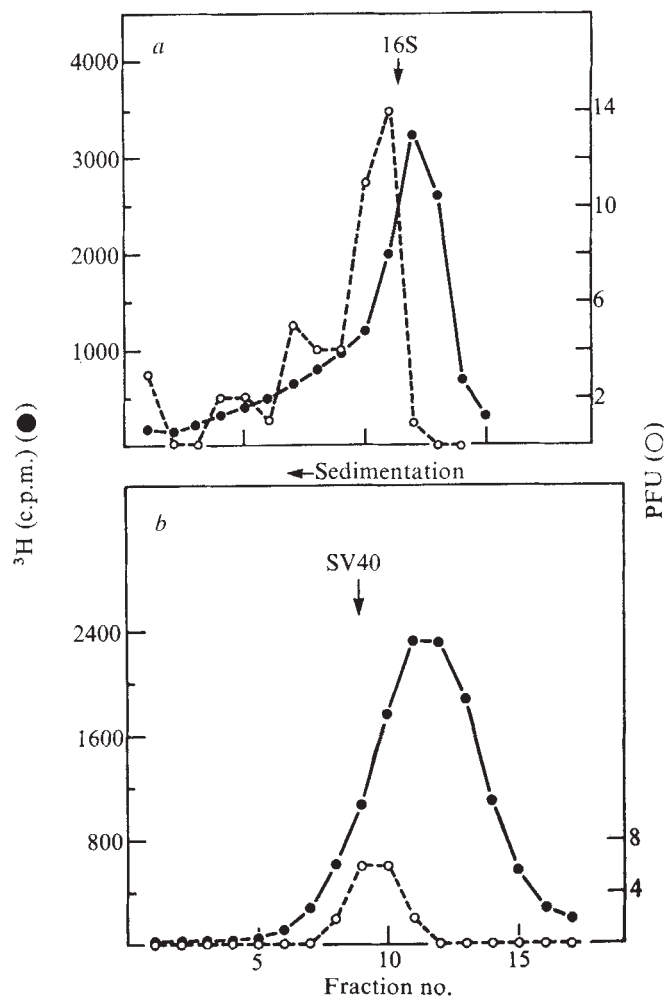


Fig. 4 Sucrose gradient sedimentation of reverse transcriptase product. *a*, Total nucleic acid from an endogenous reaction was centrifuged through a 4.8-ml 15–30% sucrose gradient in 0.1 M NaCl, 0.02 M Tris-HCl, pH 8.3, 0.002 M EDTA, for 2 h at 48,000 r.p.m., 4°C , in the SW 50.1 rotor. The 16S marker, ^{14}C -labelled SV40 DNA cleaved with *Eco*RI endonuclease, was centrifuged in a parallel bucket. Fractions of 0.32 ml were collected from the bottom and the ^3H -labelled DNA was detected by liquid scintillation counting of 30 μl aliquots ($\bullet$). Each fraction was used to transfect a single plate of NIH/3T3 cells ($\circ$). *b*, A sample of one of the DNA preparations described in the legend to Fig. 2 was centrifuged through a 5-ml gradient of 5–20% sucrose in 0.3 M NaOH, 0.7 M NaCl, 0.005 M EDTA, 0.1% sarkosyl, for 4.5 h at 49,000 r.p.m., 4°C , in the SW 50.1 rotor. Fractions of 0.26 ml were collected from the bottom, neutralised with an equal volume of 100 mM Tris, 0.3 M acetic acid, and used to transfect single plates of cells as above ($\circ$, PFU). A portion of the unfractionated DNA was transfected in parallel, yielding a value for the input infectivity of 150 PFU. The DNA was detected by counting 50 μl portions of the neutralised fractions before ethanol precipitation ($\bullet$, ^3H). The marker was as described in (a). In both experiments the recovery of DNA after ethanol precipitation was greater than 80% in every fraction.

fragment might be necessary as a primer for cellular DNA polymerase to complete the proviral DNA duplex before integration.

This interpretation does not directly address the question of the unexpected size difference between the longest strands made in the presence and absence of actinomycin D. If the longest DNA made in the presence of the drug were in fact a complete transcript, then the extra sequences might be part of a self-complementary 'hairpin' structure initiating the positive strand. Alternatively, the additional sequences might represent a transcript of the 5'-proximal 600 nucleotides of the RNA template, untranscribed in the presence of the inhibitor.

In summary, we have shown that detergent-disrupted virions of M-MuLV can synthesise DNA which is infectious. DNA made in the endogenous reverse transcriptase reaction can specify the synthesis of complete, infectious virus with the host range of the parent stock; this genetic information is probably carried on individual duplex molecules and not provided by complementation among partial transcripts. Thus it is likely that the virions contain all activities necessary for the synthesis of unintegrated proviral DNA. Reverse transcription in purified virions may accurately reflect the mechanism of synthesis of the viral DNA in infected cells.

A preliminary account of these findings was presented at the Cold Spring Harbor meeting on RNA tumour Viruses, May 30, 1976. We would like to thank Daniel Donoghue for thoughtful advice and valuable criticism throughout this work. This research

was supported by grants from the National Cancer Institute and the American Cancer Society and by a contract from the Virus Cancer Program of the National Cancer Institute. During part of this work, E.R. was supported by an NSF graduate fellowship. R.A.W. is a Cancer Research Scholar of the American Cancer Society, Massachusetts Division. D.B. is a Research Professor of the American Cancer Society.

Received 5 May; accepted 20 June 1977.

- ¹ Baltimore, D. *Nature* **226**, 1209–1211 (1970).
- ² Temin, H. M. & Mizutani, S. *Nature* **226**, 1211–1213 (1970).
- ³ Boettiger, D. & Temin, H. M. *Nature* **228**, 622–624 (1970).
- ⁴ Balduzzi, P. & Morgan, H. R. *J. Virol.* **5**, 470–477 (1970).
- ⁵ Junghans, R. P., Duesberg, P. H. & Knight, C. A. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4895–4899 (1975).
- ⁶ Rothenberg, E. & Baltimore, D. *J. Virol.* **21**, 168–178 (1977).
- ⁷ Hu, S., Davidson, N. & Verma, I. M. *Cell* **10**, 469–477 (1977).
- ⁸ Haseltine, W. A., Kleid, D. G., Panet, A., Rothenberg, E. & Baltimore, D. *J. molec. Biol.* **106**, 109–131 (1976).
- ⁹ Peters, G., Harada, F., Dahlberg, J. E., Panet, A., Haseltine, W. A. & Baltimore, D. *J. Virol.* **21**, 1031–1041 (1977).
- ¹⁰ Graham, F. L. & van der Eb, A. J. *Virology* **52**, 456–467 (1973).
- ¹¹ Graham, F. L. & van der Eb, A. J. *Virology* **54**, 536–539 (1973).
- ¹² Smolkin, D., Gianni, A. M., Rozenblatt, S. & Weinberg, R. A. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4910–4913 (1975).
- ¹³ Smolkin, D., Yoshimura, F. K. & Weinberg, R. A. *J. Virol.* **20**, 621–626 (1976).
- ¹⁴ Rowe, W. P., Pugh, W. E. & Hartley, J. *Virology* **42**, 1136–1139 (1970).
- ¹⁵ Levy, J. A. *Science* **182**, 1151–1153 (1973).
- ¹⁶ Birnie, G. D. *FEBS Lett.* **26**, 19–22 (1972).
- ¹⁷ Manly, K. F., Smoler, D. F., Bromfield, E. & Baltimore, D. *J. Virol.* **7**, 106–111 (1971).
- ¹⁸ Garapin, A.-C., Varmus, H. E., Faras, A. J., Levinson, W. E. & Bishop, J. M. *Virology* **52**, 264–274 (1973).
- ¹⁹ McDonnell, M. W., Simon, M. N. & Studier, F. W. *J. molec. Biol.* **110**, 119–146 (1977).
- ²⁰ Gianni, A. M. & Weinberg, R. A. *Nature* **255**, 646–648 (1975).
- ²¹ Fritsch, E. & Temin, H. M. *J. Virol.* **21**, 119–130 (1977).
- ²² Sharp, P. A., Sugden, B. & Sambrook, J. *Biochemistry* **12**, 3055–3063 (1973).
- ²³ Lasky, R. A., Mills, A. D. & Knowland, J. S. *Eur. J. Biochem.* **56**, 335–341 (1975).

letters to nature

Pulsar interpulses and other off-pulse emission

THE pulse emission from pulsars is usually confined to a window having a width of a few per cent of the pulsar period. Seven out of the 149 known pulsars, however, exhibit secondary pulses known as interpulses¹ which follow the relatively stronger main pulses by about half of the pulsar period. While it seems that only a few pulsars have observable interpulses, not all of the known pulsars have been carefully examined for this phenomenon. This is mainly because once a pulsar has been discovered, observers tend to concentrate on studies of the pulse window rather than the whole pulsar period. Consequently it would be statistically incorrect to compare the fraction of pulsars known to have interpulses with the fraction predicted by a proposed pulsar model. In order to check this possibility we conducted an interpulse search on the pulsars that are visible from Jodrell Bank. We report here the results of this search in which one new interpulse was discovered. These observations also revealed the presence of weak emission from regions other than the main pulse and interpulse windows in the cases of three pulsars.

The search took place between July 1975 and April 1976. Observations were made on approximately 100 pulsars at 327, 408 and 610 MHz and of about 50 of these pulsars at 151 and 240 MHz. For each pulsar the full period was observed for a total time of about 90 min. Dispersion removing techniques² were used to keep dispersion smearing comparable with the bin size of 1/400th of the pulsar period. At each frequency observations were made simultaneously, using two contiguous frequency bands of widths 2 MHz at 408 MHz and 610 MHz and 0.5 MHz, at the other frequencies. This enabled an interpulse to be distinguished from interference since the pulse arrival

times would be different in the two bands due to dispersion. The observation of an interpulse at several frequencies provided further checks on the validity of an identification.

Out of all the pulsars observed only PSR1822–09 revealed a previously unknown interpulse. The full period profile of this pulsar is shown in Fig. 1. The interpulse, whose intensity is ~10% of the main pulse, has been seen at 327, 408 and 610 MHz with approximately 170° of longitude separation from the main pulse (where 360° represents the whole pulsar period). Note that PSR1822–09 has several features in common with the Crab Nebula pulsar, PSR0531+21. Besides having interpulses both pulsars have a precursor to the main pulse and both have relatively large values of *P* (the period time derivative). Observations³ of PSR1822–09 indicate that this pulsar also emits pulsed γ rays like PSR0531+21. We suggest that there may be a causal connection between these phenomena.

Figure 1 also shows the profile of PSR0740–28 for which a new component has been detected at 240 MHz. This component has ~4% of the intensity of the main pulse and follows the main pulse by about 70° of longitude. The component is below the limit of detection at 327 MHz and is obscured by interstellar scattering at 151 MHz. The separation between this component and the main pulse is possibly too small to identify it as an interpulse and we regard it as part of the main pulse window which, therefore, has a width of about 70° longitude.

From the survey, therefore, it seems that interpulses are a property of only a few pulsars; of the 105 pulsars studied only four, PSR0531+21, PSR0950+08, PSR1822–09 and PSR1929+10, showed interpulses. Of the remaining pulsars with known radio interpulses, PSR0904+77, PSR0823+26 and PSR1055–52, two were too weak to be detected and one is not visible from Jodrell Bank. (The interpulse in the Vela pulsar, PSR0833–45, is only seen at optical and γ wavelengths). The pulsars with interpulses have, with one exception (PSR0904

+77), shorter than average periods and three of the six that have determined values of $\dot{P}$ have very large values for this. These are thought to be characteristic of young pulsars⁴ which suggests that interpulses may also be a property of young neutron stars. This accords with theory since interpulses are generally thought to indicate that the observer is seeing the effects of both magnetic poles of the neutron star. This occurs when the magnetic axis is almost orthogonal to the rotation axis of the star and Jones⁵ has suggested that this may in fact be the case for the youngest pulsars. According to this model the axes begin to realign after $\sim 10^6$ yr and consequently interpulses should not be seen for the older pulsars. A completely opposite view of pulsar magnetic field alignment has been put forward by Flowers and Ruderman⁶. They also propose an alternative explanation of interpulses and our results may be understood in terms of either model.

Our observations also provided a second result: three pulsars exhibited low frequency emission with a wide longitudinal distribution. Figure 2 shows the full period profiles of these pulsars at several frequencies. This weak emission was confirmed by the presence of a time delay between its components in the two adjacent frequency bands caused by dispersion. Observations⁷⁻⁹ at other wavelengths have shown similar features to be present in other pulsars but greatly enhanced. From Fig. 2 we deduce that this emission has a steeper spectrum than the main pulse radiation. This fact and the wide distribution in longitude suggest that we are dealing with a different part of

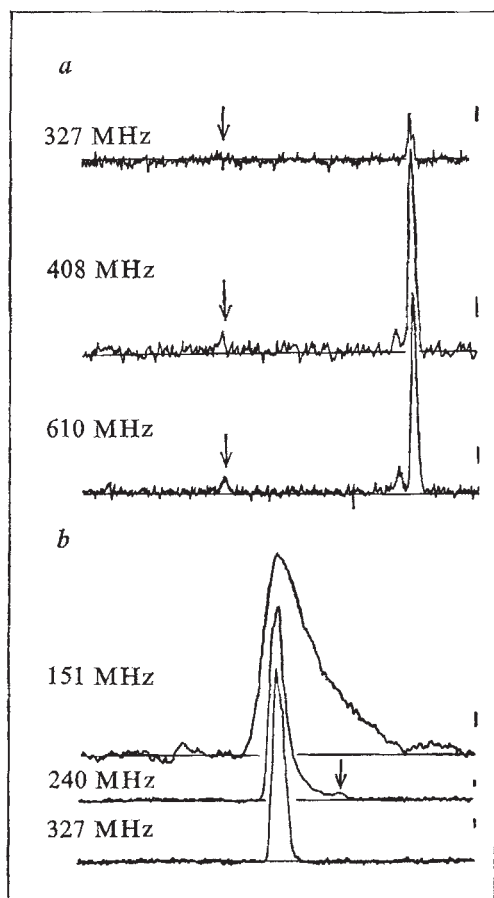


Fig. 1 Integrated profiles of *a*, PSR1822-09 and *b*, PSR0740-28. The positions of newly discovered interpulse and pulse components are marked by arrows. The intensity scale is linear but in arbitrary units and the length of the vertical bar at the top right hand of each figure represents the r.m.s. noise of the observation and corresponds to the uncertainty of the position of the fitted baseline. The width of the vertical bar shows the time resolution.

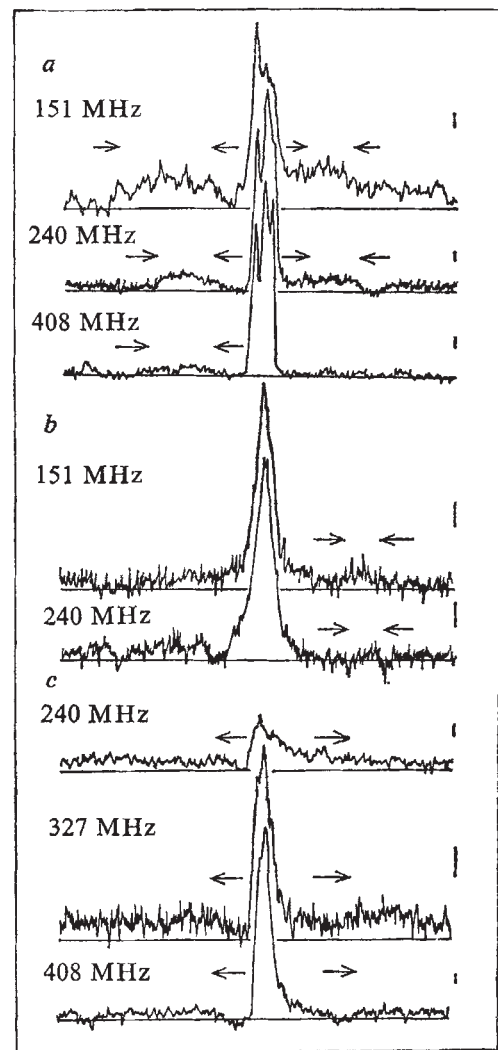


Fig. 2 The integrated profiles of three pulsars exhibiting low frequency emission with a broad longitude distribution: *a*, PSR0450-18; *b*, PSR1541+09; *c*, PSR1718-32. The extent of the emission is shown by delimiters.

the magnetosphere to that responsible for the main and interpulse emissions which tend to have similar widths and spectra.

Future observations of the polarisation of this phenomenon may help distinguish between these two emission regions and reveal further information about the magnetospheric structure of pulsars.

We thank Dr A. G. Lyne for much help and we acknowledge financial support from the SRC.

E. B. CADY
R. T. RITCHINGS*

University of Manchester,
Nuffield Radio Astronomy Laboratories,
Jodrell Bank,
Macclesfield,
Cheshire, UK

Received 15 July; accepted 26 July 1977.

*Present address: Department of Medical Biophysics, University of Manchester, UK.

- ¹ Manchester, R. N. & Lyne, A. G. *Mon. Not. R. astr. Soc.* (in the press).
- ² Lyne, A. G. & Thorne, D. J. *Mon. Not. R. astr. Soc.* 172, 97 (1975).
- ³ Kanbach, G. *et al. Proc. 12th ESLAB. Symp.* (1977).
- ⁴ Lyne, A. G., Ritchings, R. T. & Smith, F. G. *Mon. Not. R. astr. Soc.* 171, 579 (1975).
- ⁵ Jones, P. B. *Mon. Not. R. astr. Soc.* 178, 87 (1977).
- ⁶ Flowers, E. & Ruderman, M. A. *Astrophys. J.* 215, 302 (1977).
- ⁷ Bruck, Y. M. & Ustimenko, B. Y. *Nature phys. Sci.* 242, 58 (1973).
- ⁸ Bruck, Y. M. & Ustimenko, B. Y. *Nature* 260, 766 (1976).
- ⁹ Backer, B. C., Boriakoff, V. & Manchester, R. N. *Nature phys. Sci.* 243, 77 (1973).

Observable gravitational effects on polarised radiation coming from near a black hole

THE effects of general relativity on the degree and plane of linear polarisation of X rays emitted by an accretion disk orbiting a black hole (such as Cyg X-1 might contain), were described in a previous letter¹ where we showed that the effects were large. Using a constant of motion along null geodesics in the Kerr metric, discovered by Penrose and Walker², we have since been able to calculate analytically polarisation rotations along any null ray. We present here more extensive results obtained by this method, for two different kinds of disk model and for different black hole angular momenta.

The alteration in observed polarisation properties from their Newtonian values³⁻⁶ is due to the strong bending of light rays coming from the accretion disk. The effects of gravitational redshifts and focusing are to strongly 'pick-out' some regions of the disk to contribute most to the observed properties. To show these effects we have made calculations for 'one'- and 'two'- temperature disk models^{7,8}. These models differ in that the former has a radial temperature gradient, whereas in the latter the inner region, closest to the black hole, has an essentially constant temperature. Although no completely satisfactory disk model exists, our calculations give some idea of the effects to be expected from more refined models.

Figure 1 shows the variation of the plane of polarisation, ϕ , as a function of observing energy for the one-temperature model, seen at a polar angle θ_0 of 41° . An observer above the disk ($\theta_0 < 90^\circ$) sees a clockwise rotation (not anticlockwise as

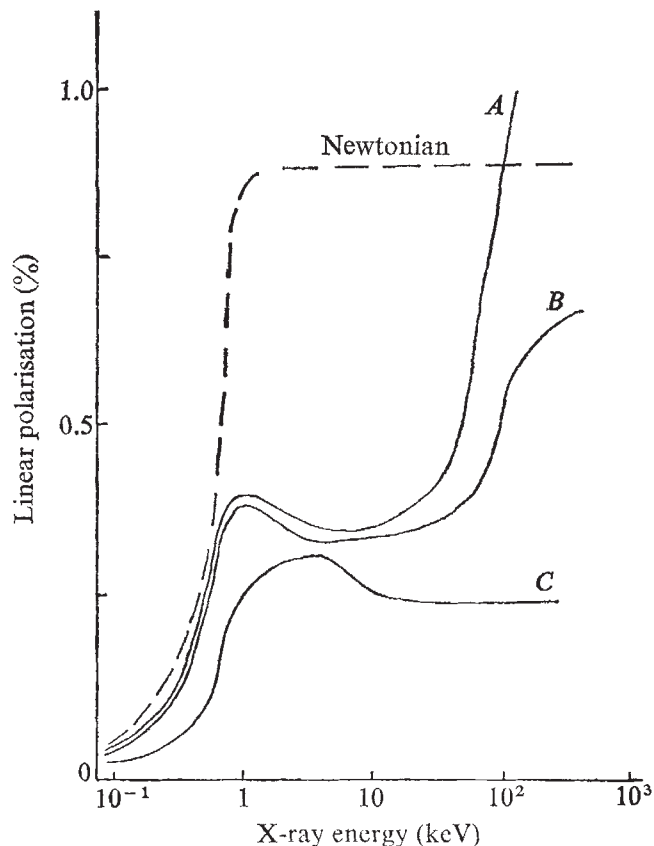


Fig. 2 Variation of linear polarisation with energy for the one-temperature model, with A, $a/m = 0.9981$; B, $a/m = 0.9$; C, $a/m = 0$. Same parameters as Fig. 1.

we stated previously¹). There is still an effect when the black hole is non-rotating ($a/m = 0$). This is because the disk itself is rotating, and the 'pick-out' still operates. Figure 2 shows the degree of polarisation for the same parameters.

The two-temperature model has an outer region with the same structure as the one-temperature model, but it becomes optically thin and hot at a transition radius of $r \lesssim 50$ gravitational radii. Above a threshold energy the radiation from the inner region is, locally, rotated by 90° compared with the optically thick case. So classically one would expect the polarisation vectors of photons coming from the outer and inner regions to be seen as perpendicular. When general-relativistic effects are included, and the 'pick-out' effect is integrated over the inner region, the observed plane of polarisation is not aligned with the projected polar axis, but has an additional jump, of the order of 10° , and the plane then has a constant direction at higher energies. Figure 3 shows the value of this jump in angle as a function of a/m , the angular momentum parameter.

Individual polarisation rotations can be determined analytically using the constant of motion found by Penrose and Walker. This is a complex number, constant along null geodesics in type (2, 2) space-times (the most astrophysically relevant of which is the Kerr metric). Its modulus is the constant of motion found by Carter⁹, and its argument contains the required information about polarisation rotations. The constant is

$$K_{pw} = \kappa^2 f^b (l_a n_b - m_l \bar{m}_b) \Psi_2^{-1/3}$$

where κ^a is the photon wavevector, f^b is the polarisation vector, $(l^a, n^a, m^a, \bar{m}^a)$ is the null tetrad based on the repeated principal null directions of the Kerr metric¹⁰, and Ψ_2 is a complex

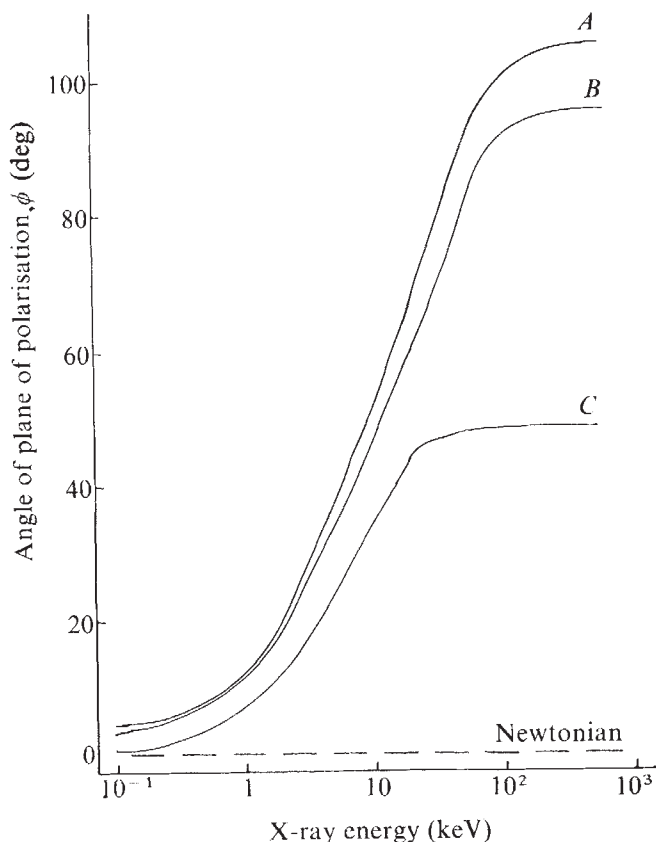


Fig. 1 Variation of plane of polarisation with energy for $\theta_0 = 41.1$ for the one-temperature model with A, $a/m = 0.9981$; B, $a/m = 0.9$; C, $a/m = 0$, where viscosity parameter, $\alpha = 0.1$; mass of black hole, $M = 9M_\odot$; mass accretion rate $= 7 \times 10^{17} \text{ g s}^{-1}$.

function coming from the Weyl tensor. In Kerr we find (using Boyer–Lindquist coordinates¹¹)

$$K_{pw} = (\alpha - i\beta)(r - ia \cos\theta) = K_2 - K_1$$

where $\alpha = (\kappa^0 f^1 - \kappa^1 f^0) + a \sin^2\theta(\kappa^1 f^3 - \kappa^3 f^1)$

and $\beta = (r^2 + a^2) \sin\theta(\kappa^3 f^2 - \kappa^2 f^3) - a \sin\theta(\kappa^0 f^2 - \kappa^2 f^0)$

Also we have $\mathbf{k} \cdot \mathbf{f} = 0$, and $\mathbf{f}$ is only determined to within a multiple of $\mathbf{k}$ (so we can choose $f^0 = 0$ at any fixed point).

For a given disk model we can evaluate α and β at the point where a null ray leaves the disk, from a knowledge of the initial polarisation vector. We then have to solve three linear equations for the three space-like components of $\mathbf{f}$ at infinity. We find for orthonormal components

$$f^r(\infty) = 0, \quad f^\theta(\infty) = (SK_1 - TK_2)/(S^2 + T^2)$$

$$f^\Phi(\infty) = (-SK_2 - TK_1)/(S^2 + T^2)$$

where $S = (L_z/\sin\theta_0 - a \sin\theta_0)$

and $T = \text{sgn}(k^\theta)_x(Q - L_z^2 \cot^2\theta_0 + a^2 \cos^2\theta_0)^{1/2}$

here Q and L_z are constants of motion along the ray. In this way we obtain, after integration over the disk, the results shown in Fig. 1.

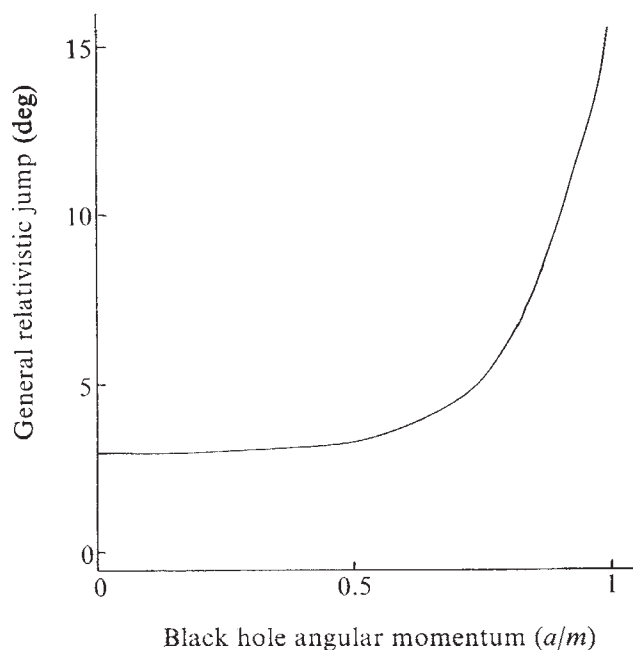


Fig. 3 General-relativistic jump for the two-temperature model, as a function of black hole angular momentum. Same parameters as Fig. 1.

Our previous numerical results for ϕ differ from the exact values by only about 1%.

We thank Roger Penrose for pointing out the analytic constant. Dennis Sciama for his help, and Drs T. Piran, G. Bath, J. Miller and Mr S. J. Rose and D. Pooler for helpful suggestions.

P. A. CONNORS
R. F. STARK

Department of Astrophysics,
South Parks Road,
Oxford, UK

Received 23 June; accepted 22 July 1977.

¹ Stark, R. F. & Connors, P. A. *Nature* **266**, 429–430 (1977).

² Walker, M. & Penrose, R. *Commun. math. Phys.* **18**, 265–274 (1970).

³ Chandrasekhar, S. *Radiative Transfer*, 219–224 (Dover, New York, 1969).

⁴ Angel, J. R. P. *Astrophys. J.* **158**, 219–224 (1969).

⁵ Lightman, A. P. & Shapiro, S. L. *Astrophys. J. Lett.* **198**, L73–L75 (1975).

⁶ Lightman, A. P. & Shapiro, S. L. *Astrophys. J.* **203**, 701–703 (1976).

⁷ Novikov, I. D. & Thorne, K. S. *Les Astres Occlus* (Gordon and Breach, New York, 1973).

⁸ Shapiro, S. L., Lightman, A. P. & Eardley, D. M. *Astrophys. J.* **204**, 187–199 (1976).

⁹ Carter, B. *Phys. Rev.* **174**, 1559–1571 (1968).

¹⁰ Breuer, R. A. *Lecture Notes in Physics* **44**, (Springer, Berlin, 1975).

¹¹ Misner, C. W., Thorne, K. S. & Wheeler, J. A. *Gravitation* (Freeman, San Francisco, 1973).

Quantum uncertainty in the final state of gravitational collapse

THE ratio of the action S to $\hbar$ (Planck's constant/ 2π) determines whether the physical system in question is to be treated classically or quantum mechanically. In the area of classical physics the ratio $S/\hbar$ is large compared with unity, and the governing equations are given by $\delta S = 0$. Quantum mechanics begins to be important when $S \lesssim \hbar$, and the definitive approach of classical physics is replaced by quantum uncertainty. We discuss here the behaviour of a physical system which is initially in the classical domain ($S \gg \hbar$) but whose later development may well take it into the region of quantum uncertainty. We consider a specific example of this—the gravitational collapse of a spherical dust ball. While classically such a dust ball ends up in a space–time singularity, the corresponding quantum mechanical result suggests a range of final states some of which are non-singular.

The explicit solution obtained here ignores pressures which have an important role in the gravitational equilibrium of stars. But classical relativity predicts that a space–time singularity develops in a body undergoing gravitational collapse in a fairly general and ‘reasonable’ set of physical conditions^{1,2}. Thus pressures satisfying ‘physically reasonable’ equations of state are not able to prevent this fate for those stars which become black holes towards the end of their evolution. For such stars S eventually becomes small enough for the above quantum considerations to become important. In these cases the above solution gives a qualitative indication of the probable types of final states.

The classical relativistic equations are derived from the variation of an action given by

$$S = \frac{c^4}{16\pi G} \int_V R(-g)^{1/2} d^4x - \sum_a \int m_a da \quad (1)$$

where c is the speed of light, G the gravitational constant, R the scalar curvature of space–time, g the determinant of the metric tensor g_{ik} . The second term of S is the action describing the behaviour of a system of particles labelled by a , with masses m_a . We may add other terms to S to include other possible physical interactions like electromagnetism. V is the 4-volume of space–time under consideration. In the classical approach, we may think of V as the region sandwiched between two chronologically ordered spacelike hypersurfaces Σ_1 and Σ_2 . The specification on Σ_1 of a 3-geometry $^{(3)}G_1$, and $^{(3)}G_2$, together with the field equations obtained from $\delta S = 0$ would lead to the determination of the 3-geometry $^{(3)}G_2$ on Σ_2 (see ref. 3).

The classical approach is a good one so long as $S \gg \hbar$, where $2\pi\hbar = h$ = Planck's constant. For most situations discussed in general relativity this condition is satisfied. But in the gravitational collapse of a compact object S may become small enough to be of the order $\hbar$ close to the singularity. It therefore becomes necessary to examine this problem from the quantum mechanical point of view. Here we adopt the path integral approach.

In this approach, non-classical geometries, that is, those not satisfying $\delta S = 0$ are permitted and the above sandwich problem is rephrased thus. Given two 3-geometries, $^{(3)}G_1$ on Σ_1 and $^{(3)}G_2$ on Σ_2 what is the probability amplitude for the system with the given action (1) to go from one to the other? The answer may be expressed as a propagator $K(^{(3)}G_2, \Sigma_2; ^{(3)}G_1, \Sigma_1)$ which is obtained by the Feynman rule of sum over histories⁴.

In practice the execution of this project is extremely difficult and has not been done in the general situation. It is, however, possible to obtain an exact solution under a very limited form. Since this

form gives an insight into the gravitational collapse problem, it is described below in brief.

Following DeWitt⁵ we take into consideration only the conformal degrees of freedom. Suppose the classical solution of $\delta S = 0$ is described by a metric $\bar{g}_{ik}$. Consider non-classical solutions which are conformal to the classical one

$$g_{ik} = \Omega^2 \bar{g}_{ik} \quad (2)$$

where Ω is the conformal function. From (2) we get

$$\int_V R(-g)^{1/2} d^4x = \int_V (\Omega^2 \bar{R} - 6\Omega_i \Omega^{,i})(-\bar{g})^{1/2} d^4x + \text{surface term} \quad (3)$$

where on the right hand side the quantities refer to the classical metric. In the summing over histories we now have only the histories of Ω from Σ_1 to Σ_2 .

The problem is further simplified for the case of a collapsing homogeneous ball of dust in an otherwise empty space-time⁶. In terms of the comoving coordinates r ($\leq r_b$), θ , Φ , t , the classical solution is given by the line element

$$d\bar{s}^2 = c^2 dt^2 - Q^2(t) \left[\frac{dr^2}{1 - \alpha r^2} + r^2(d\theta^2 + \sin^2\theta d\phi^2) \right] \quad (4)$$

in the interior of the ball. The scale factor $Q(t)$ is taken to be unity at the start of the collapse when the density was ρ_0 . The Einstein field equations $\delta S = 0$ give

$$\dot{Q}^2 = c^2 \alpha \left(\frac{1-Q}{Q} \right), \quad \alpha = \frac{8\pi G \rho_0}{3c^2} \quad (5)$$

The singularity ($Q = 0$) develops in a t -interval $\pi/(2c\alpha^{1/2})$. We will denote this instant by $t = 0$. As we are interested in the final stages of collapse, we will approximate the solution of (5) by

$$Q = \left(\frac{3c}{2} \alpha^{1/2} \right)^{2/3} T^2, \quad T = (-t)^{1/3} \quad (6)$$

Consider now the quantum fluctuations which preserve the symmetries of the problem. These are the conformal fluctuations for which Ω is a function of t only. We denote Σ_1 and Σ_2 by $t = t_1$, ($T = T_1$) and $t = t_2$, ($T = T_2$) respectively. Following the techniques of path integration⁴ it is possible to compute K exactly. The answer is best expressed in terms of the departure from the classical solution ($\Omega = 1$)

$$\Phi = \Omega - 1 \quad (7)$$

Given $\Phi = \Phi_1$ on Σ_1 and $\Phi = \Phi_2$ on Σ_2 we have

$$K(\Phi_2, T_2; \Phi_1, T_1) = \left(\frac{3iV\rho_0 c^2 T_1^2 T_2^2}{4\pi\hbar(T_1 - T_2)} \right)^{1/2} \times \\ \times \exp \left\{ \frac{3iV\rho_0 c^2}{4\hbar(T_1 - T_2)} [T_1^3(T_1 - 2T_2)\Phi_1^2 + \right. \\ \left. + (T_2 - 2T_1)T_2^3\Phi_2^2 + 2T_1^2 T_2^2 \Phi_1 \Phi_2] \right\} \quad (8)$$

where V is the coordinate volume of the ball.

This kernel has the reproducing property and its significance can be seen in the following way. Suppose at Σ_1 the state of the dust ball is described by a wave packet of dispersion Δ_1 in Φ_1

$$\Psi(\Phi_1, \Delta_1) = \left(\frac{1}{2\pi\Delta_1^2} \right)^{1/4} \exp \left(-\frac{\Phi_1^2}{4\Delta_1^2} \right) \quad (9)$$

where $\Delta_1 \ll 1$. Then at Σ_2 , with $|t_2| \ll |t_1|$, the state is described by a wave packet with a mean $\langle \Phi_2 \rangle = 0$ and a dispersion

$$\Delta_2 = \left(\frac{\hbar}{3V\rho_0 c^2 T_1 \Delta_1} \right) \left\{ 1 + \left(\frac{3V\rho_0 \Delta_1^2 c^2}{\hbar} \right)^2 T_1^6 \right\}^{1/2} T_2^{-2} \quad (10)$$

Thus, however small Δ_1 may be, equation (10) shows that Δ_2 diverges as $T_2 \rightarrow 0$, ($t_2 \rightarrow 0$). In other words, although the wave function at t_2 continues to have the classical solution ($\Phi_2 = 0$) as the mean, the spread around this solution gets progressively larger as the so-called classical singular epoch is approached: (10) effectively tells us where the quantum uncertainty begins to dominate. For a solar mass dust ball with $\rho_0 = 1 \text{ g cm}^{-3}$, the linear shrinkage must factor 10^{-43} before the quantum uncertainty takes over.

Although the smallness of this ratio indicates the range over which the classical theory is valid, the ultimate dominance of quantum uncertainty as $t \rightarrow 0$ seems inescapable.

This solution can also be used to discuss the early stages of a Friedmann universe. A few years ago Hoyle and Narlikar⁷ had suggested that the quantum fluctuations in a classical big bang model might produce models without particle horizons (which inhibit the transfer of information). It is interesting to note that the range of uncertainty indicated by Δ_2 permits such models.

I thank Professor J. A. Wheeler for discussions and for hospitality at the Center for Theoretical Physics, University of Texas at Austin. I also thank Professor Cecile DeWitt for discussions on path integrals, and the International Astronomical Union for a travel grant.

J. V. NARLIKAR*

Center for Theoretical Physics
University of Texas at Austin
Austin, Texas 78712

Received 9 May; accepted 23 June 1977.

*Permanent address: Tata Institute of Fundamental Research, Bombay 400 005, India.

¹ Penrose, R. *Phys. Rev. Lett.* **14**, 57 (1965).

² Hawking, S. W. & Ellis, G. F. R. *The Large Scale Structure of Space time* (Cambridge University Press, Cambridge, 1973).

³ Misner, C. W., Thorne, K. S. & Wheeler, J. A. *Gravitation* (Freeman, San Francisco, 1973).

⁴ Feynman, R. P. & Hibbs, A. R. *Quantum Mechanics and Path Integrals* (McGraw-Hill, New York, 1965).

⁵ DeWitt, B. S. *Phys. Rev.* **162**, 1239 (1967).

⁶ Hoyle, F. & Narlikar, V. V. *Proc. R. Soc. A* **278**, 465 (1964).

⁷ Hoyle, F. & Narlikar, J. V. *Nature* **228**, 544 (1970).

Origin of diffuse interstellar lines

SINCE their discovery over 40 years ago, the origin of the diffuse interstellar absorption lines has been a mystery although there have been many suggestions put forward. Developments in spectroscopy and astronomy have intensified rather than reduced the mystery. This letter takes into account some of these recent developments and suggests a rather definite (but unconfirmed) identification of the lines. Excellent surveys of the diffuse interstellar lines have been given by Herbig¹ and by Wu², so the many studies of these lines will not be mentioned again. For our purposes, it is sufficient to note that the strongest line which lies at 4,428 Å has a half width of 20 Å, and that 38 weaker lines of varying strengths and widths lie at longer wavelengths. The strength of the lines is strongly correlated with regions in space containing interstellar grains (particularly small grains) and there is strong evidence that all the lines arise from a single species or from closely related species. The only feature in the spectrum lying to the violet of 4,428 Å which seems to be related to the diffuse lines is a continuum in the 2,200 Å region but this must certainly involve much higher electronic states of the absorbing species.

The line widths of the diffuse lines are the unique characteristics which differentiate them from all identified interstellar lines. The line widths which vary between 1 and

100 cm^{-1} seem to preclude assigning the lines to transitions between discrete states of free atoms or molecules (which at the temperature of the interstellar region give bands narrower than the $40\text{--}100\text{ cm}^{-1}$ widths of most of the stronger lines). Many suggestions have been made regarding the origin of the lines. The line widths have been accounted for by assuming either that the absorbing species is pre-ionised or predissociated in its excited state, or that the absorbing species is on or in an interstellar grain where site inhomogeneities account for the line widths. The first of these hypotheses suffers because in spite of numerous studies of most of the abundant simple molecules and atoms, it has not been possible to identify the absorbing species. A second difficulty with the predissociation hypothesis is that each absorbed photon destroys the absorber and an efficient process must exist to restore the population. The second hypothesis also suffers because it has not been possible to identify the species which is responsible for the absorption. More important, considering the probable wide variation of sizes and compositions of the grains, it is difficult to conceive a system of grains and absorbers which will give the observed discrete set of lines with widths down to a few cm^{-1} and give series of lines extending only to the red of the strongest line at $4,428\text{ \AA}$. Danks and Lambert³ have shown that for a certain reasonable range of molecular moments of inertia, dipole moments and collision rates, the shapes of some of the diffuse lines can be fitted by band contours of heavy molecules. They do, however, have difficulty in fitting the wider lines and probably in accounting for the wide range of bandwidths.

Here I suggest that the lines are caused by the absorption of polyatomic molecules and that the line width is the result of radiationless internal conversion between stable states. Furthermore, I propose that the absorbing species are long chain carbon molecules, C_n where n may lie in the range 5–15. The direct evidence to support this is very meagre indeed but this suggestion seems to account for the observations without straining our present concepts of spectroscopy or astronomy.

A radiationless internal conversion results from an interaction between the levels of an excited electronic state and the higher vibrational levels of a lower state⁴. The process causes each rotational line of an electronic transition to be replaced by a band whose width depends on the interaction energy. This interaction energy varies greatly from one state to another and even from one vibrational level to another in the same electronic state. The band widths can be predicted only by very elaborate calculations but widths in the $1\text{--}100\text{ cm}^{-1}$ range are not unusual. For all but the most simple molecules, the band consists of such a high density of lines that it is for all practical purposes a continuum. Following the absorption of radiation in this band, the molecule will lose its excitation through a series of vibrational transitions. Thus, in molecules displaying this type of radiationless transition, diffuse bands are not accompanied by dissociation. It is worth noting that internal conversions are not rare phenomena but exist in most polyatomic molecules and are particularly important for the larger molecules.

Recently HC_3N (ref. 5), HC_5N (ref. 6), and HC_7N (H. W. Kroto *et al.*, submitted for publication) have been identified by radio observations of dense interstellar clouds. It seems most likely that this series will be extended to at least HC_9N once the spectrum of this molecule has been identified in the laboratory and it is reasonable to assume that long chain acetylenes, which have no dipole moment and thus cannot be observed, are more abundant than the observed cyanoacetylenes. How these long chains of carbon atoms are formed is unknown although their existence is firmly established. It can, therefore, be suggested that similar long chains of carbon atoms, either formed by the same process or released from

clouds, exist in the less dense interstellar regions. In the less dense regions, these chains may lose any hydrogen they had or acquire, by photodissociation and exist as pure carbon chains. We may further postulate that the shortest of the chains (C_2 , C_3 , C_4) are photodissociated but chains beyond a certain length can lose the energy absorbed in the form of radiation by the internal conversion process and are not readily photodissociated.

Pitzer and Clementi's⁷ calculations on the structures and electronic states of long chain carbon molecules agree with the experimentally observed high stability of these molecules, and indicate that the stability of chains with odd numbers of atoms is greater than those with even numbers. These calculations, which give only a qualitative picture of the absorption spectra, indicate that the smallest of the molecules should show strong absorption bands in the 2-eV ($6,000\text{-\AA}$) region with the corresponding absorption shifting to the red with increased chain length. Some alteration between chains with odd and even numbers of atoms is also expected in the position of the absorption spectrum with the odd tending to lie to shorter wavelengths. The simplest polyatomic carbon molecule, C_3 , is known to have a strong absorption band at $4,050\text{ \AA}$ (ref. 8) and there is evidence that C_4 has absorption bands in the $5,000\text{-\AA}$ region⁹. Therefore, if we examine the spectrum of a mixture of C_n molecules in which the abundance of C_n decreases as n increases and n has a certain lower limit such as 5, we should expect to find a series of bands extending to the red from a strong band in $4,000\text{--}5,000\text{-\AA}$ region.

The facts and postulates given above suggest that the strong $4,428\text{ \AA}$ band arises from the absorption of a C_n molecule where n is a number such as 5, 7 or 9. The absorption bands at longer wavelengths result from the various absorptions of longer chains. The known correlation between these absorption bands and small interstellar grains seems reasonable since at least some fraction of these grains may be nothing more than an aggregate of carbon chains. It is possible that some of the interstellar extinction in the ultraviolet, which is usually attributed to small interstellar grains, may be due to absorption into the higher electronic states of long chain carbon molecules.

The present hypothesis has the following merits: (1) It associates the observed absorption with very stable molecules which are composed of one of the most abundant elements and which have absorption bands in the region of the observed lines. (2) It accounts for the absence of lines at wavelengths shorter than the strong $4,428\text{-\AA}$ band. (3) It presents a process which could give the observed line widths and which does not involve the dissociation of a molecule with the absorption of each photon. (4) There is evidence that long chains of carbon atoms exist in at least some interstellar regions. (5) The association of the absorption bands with small interstellar grains seems natural.

The weakness of this suggestion lies in the lack of experimental evidence for the several postulated mechanisms. It has been assumed that radiationless transitions between stable states account for the line widths of the diffuse lines and may also protect the long chain molecules from photodissociation by ultraviolet light. It has been assumed that photodissociation destroys the short chain carbon molecules and removes other atoms, particularly hydrogen, from the chains and it has been assumed that the absorption bands of long chain carbon molecules lie at the positions of the observed diffuse lines. It may be possible to devise experimental test for some of these assumptions. Thompson, DeKock and Weltner¹⁰ have observed the infrared spectra of several long chain carbon molecules in argon matrices and there have been several mass spectrometry studies¹¹. Experimental studies of the electronic spectra of such carbon molecules should be possible in the near future.

I thank Drs G. Herzberg, J. M. MacLeod and T. Oka for their helpful comments.

A. E. DOUGLAS

*Herzberg Institute of Astrophysics,
National Research Council,
Ottawa, Ontario, Canada*

Received 18 April; accepted 13 July 1977.

- ¹ Herbig, G. H. *Astrophys. J.* **196**, 129–160 (1975).
- ² Wu, C. *Astrophys. J.* **178**, 681–699 (1972).
- ³ Danks, A. C. & Lambert, D. L. *Mon. Not. R. astr. Soc.* **174**, 571–586 (1976).
- ⁴ Jortner, J., Rice, S. A. & Hochstrasser, R. M. *Adv. Photochem.* **7**, 149–309 (1969).
- ⁵ Turner, B. E. *Astrophys. J.* **163**, L35–L39 (1971).
- ⁶ Avery, L. W., Broten, N. W., MacLeod, J. M., Oka, T. & Kroto, H. W. *Astrophys. J.* **205**, L173–L175 (1976).
- ⁷ Pitzer, K. S. & Clementi, E., *J. Am. chem. Soc.* **81**, 4477–4485 (1959).
- ⁸ Gausset, L., Herzberg, G., Lagerqvist, A. & Rosen, R. *Astrophys. J.* **142**, 45–76 (1965).
- ⁹ Graham, W. R. M., Dismuke, K. I. & Weltner, W. *Astrophys. J.* **204**, 301–310 (1976).
- ¹⁰ Thompson, K. R., DeKock, R. L. & Weltner, W. *J. Am. chem. Soc.* **93**, 4688–4695 (1971).
- ¹¹ Berkowitz, J. & Chupka, W. A. *J. chem. Phys.* **40**, 2735–2736 (1964).

Carbonaceous compounds in interstellar dust

SEVERAL infrared bands found recently in astronomical sources have not been assigned to specific substances in a convincing way (see refs 1–6). Resonance features in solids seem to be the most plausible emission and absorption mechanisms, but the features cannot be identified with known or suspected compounds in the interstellar dust grains such as silicates, water ice, silicon carbide, or graphite. We propose here a new substance, consisting of carbonaceous material, which may be responsible for some or all of the unidentified infrared features.

The infrared bands for which there are no positive identifications, or for which the identifications rest on the assignment of a single band, are listed in Table 1. The band at 3.3 μm has been studied in greatest detail. It is definitely broader than a single emission line and is unresolved at a spectral resolution of $\lambda/\Delta\lambda \sim 3,000$, ruling out the most attractive molecular candidates for the feature⁶. The apparent temperature independence of the feature and its presence in sources with widely differing physical conditions also makes a molecular origin seem doubtful. Here we interpret all of the features in Table 1 as solid resonance bands.

References to the other emission bands are given in Table 1. Less than ten sources containing these features have been observed so correlations among them are not yet firmly established. It seems that the presence of the 3.3- μm band and the 11.3- μm bands is not correlated, but the presence of the 3.4- μm and 11.3- μm bands might be⁶. Emission features at 6.2 μm and 7.7 μm were discovered by Russell *et al.*⁵ in the spectrum of the planetary nebula, NGC 7027. Observations at these wavelengths are just beginning to become available, and the sparsity of sources at this time is merely an observational effect. We have also listed the band at 11.3 μm as 'unidentified' despite previous suggestions of a carbonate origin, since subsequent observations did not reveal expected carbonate features

at 7 μm and between 28 and 35 μm (refs 1, 4). Therefore this identification must still be regarded as provisional.

Strong emission near 3.3 μm is highly suggestive of a substance incorporating carbon–hydrogen bonding since this is the well known wavelength of the fundamental C–H stretching vibration. Absorption bands between 3 and 3.5 μm are observed in carbon stars and some of these have recently been identified as HCN and C₂H₂ by high-resolution spectroscopy⁷. The existence of organic molecules which may participate in particle formation near carbon-rich objects is therefore well established. It is also plausible that in some cases the C–H bond is associated with a solid particle. The C–H could, for example, be attached to a matrix such as the observed silicate particles or the conjectured graphite grains. It is unlikely, however, that this is the case for all the sources since silicate emission or absorption does not necessarily accompany the unidentified bands. A more likely configuration would be a particle composed largely or entirely of carbonaceous material in which the C–H bonds are an ubiquitous component in an assemblage of organic molecules. Theoretical studies indicate that in a carbon-rich atmosphere, molecules such as C₂H₂ would condense to form a graphite-like substance or carbon particles which would contain at least a small admixture of C–H bonded material^{8,9}.

Grains formed in stellar atmospheres and expelled into a circumstellar environment, planetary nebula, or the interstellar medium would have a complex nucleation and growth history as well as a complex history of interaction with radiation and particles. While the resulting composition and structure of such grains is very uncertain, it seems plausible that they would be composed of a mixture of a large number of compounds reflecting the many chemical and physical processes to which the material has been subjected. For grains forming in a carbon-rich environment, one would then expect an ill-defined substance of high-molecular weight compounds with polymer or tar-like properties. Note, however, that the 3.3- μm band should be present in almost all organic compounds or combinations of them which condense to form solid particles.

The material we propose would resemble the high molecular weight organic compounds found in the carbonaceous chondrites. The major organic component in carbonaceous chondrites is a poorly defined substance which is apparently difficult to study in detail. The material is often called a 'polymer', but it is in fact not made of repeating monomers and seems to be an agglomeration of various units arranged in complicated ways¹⁰. This polymer-type material makes up 90% to 95% of the organic matter in meteorites. The hydrocarbons and amino acids in the chondrites are included in the soluble 5–10% of the organic matter.

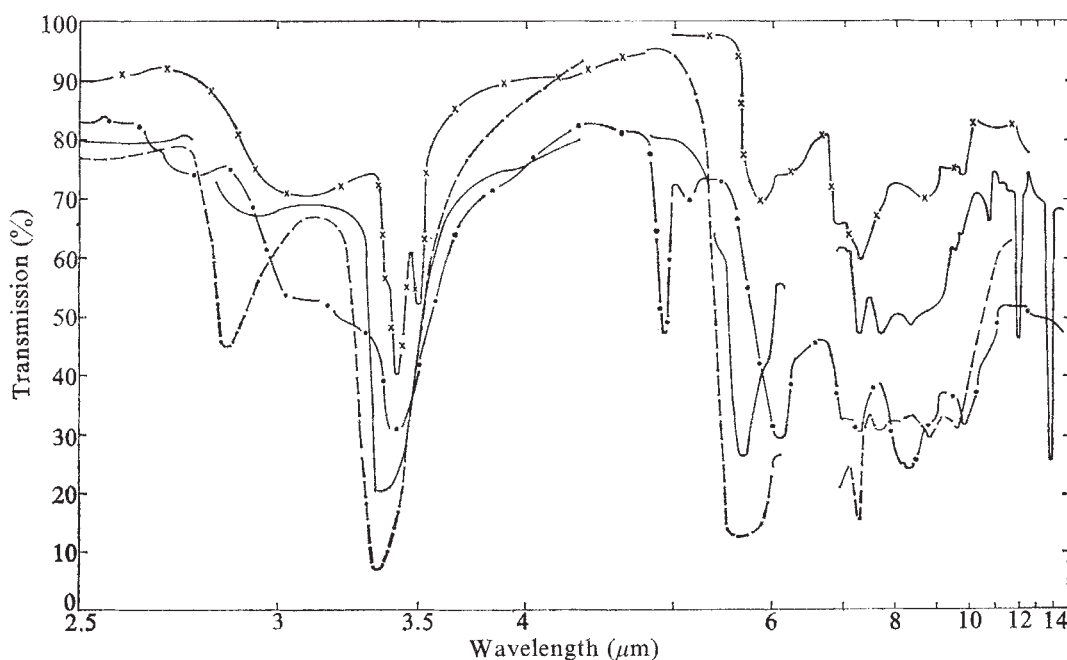
There may be further similarities with organic material synthesised in conditions resembling planetary atmospheres. Methane and ammonia, have been subjected to electrical discharges¹¹, ultraviolet radiation¹², and proton irradiation¹³. In all cases high molecular weight, polymer-like solid material is formed. Khare and Sagan pointed out the similarities between the synthesised material and the organic compounds in carbonaceous chondrites¹². This material may, however, differ in substantial ways from condensation products in stellar atmospheres where conditions do not resemble those in the experiments or in the solar nebula. In this case, the present comparison is quite qualitative and based on the assumption, suggested by the arguments above, that similar carbonaceous material might form in disparate environments. On the other hand, circumstellar shells surrounding protostars are also likely formation sites for interstellar grains¹⁴. Grains formed there might resemble the carbonaceous meteoritic material quite closely.

The infrared spectra of some of these compounds are shown in Fig. 1 which includes spectra typical of the

Table 1 Unidentified infrared emission bands

Wavelength (μm)	Objects	Refs
3.3	Planetary nebulae, HII regions, galaxies	2, 3, 6
3.4	Planetary nebulae, HII regions	2, 6
6.2	Planetary nebula (NGC 7027)	5
7.7	Planetary nebula (NGC 7027)	5
8.7	Planetary nebulae	1, 17
11.3	Planetary nebulae, HII regions, galaxies	1, 4, 17

Fig. 1 Spectra of carbonaceous materials: (—) carbonaceous chondrite, Orgeil (extract), from Meinschein, *et al.*¹⁸; (---) carbonaceous chondrite, Murray (extract), from Meinschein *et al.*¹⁸; (—x—) polymeric material formed by ultraviolet irradiation of gases in reducing atmospheres, from Khare and Sagan¹⁹; (—●—) polymeric material formed by proton irradiation of gases in reducing atmospheres, from Scattergood, *et al.*¹³.



meteoritic and synthetic materials. This comparison is subject to numerous uncertainties due to differences in sample preparation in the experiments. Particularly significant is the effect of the solvents on the material and that different extracts of the materials were measured. Nevertheless the spectra provide a qualitative comparison of the infrared properties of some of the organic compounds which form in the rather different conditions used in the experiments, and which may have analogues in the interstellar dust.

All of the spectra contain the expected strong absorption near 3.3 μm . The materials tend to be transparent between 3.6 and 5.7 μm and have a strong band located somewhere between 5.8 and 6.1 μm . This is identified as the vibration of C=C (ref. 11). Since this bonding would also be expected to be common in carbonaceous grains, we suggest that the grain feature at 6.2 μm (Table 1) is a C=C vibration. There is a discrepancy in band position between the astronomical spectra and the spectra of Fig. 1 of 0.1–0.5 μm which would have to be explained by a shift between the solid and dissolved material or perhaps by particle size and shape effects. Band displacements of a few tenths of microns are frequently observed in particle spectra¹⁵.

The spectra between 6.5 and 15 μm are different from each other and from the interstellar emitter. There seem to be real differences between the synthetic and chondritic materials. In the spectrum of Russell *et al.*⁵, the 7.7- μm emission seems to extend from 7.5 to 8.5 μm . While there is an overlap with an absorption probably due to C–O vibration between 8 and 9 μm , this is not a very convincing similarity. No strong feature near 11.3 μm occurs in the laboratory spectra. Either the 11.3- μm band is not due to carbonaceous material or is it due to a polymer somewhat different from those shown in Fig. 1. In this picture, however, it has a different molecular origin than the 3.3- μm feature. Unfortunately, the nature of the absorptions in this wavelength region complicates inferences derived from the spectra. Bands near 11.3 μm do occur in some organic compounds, for example the methyl group –CH₃ has an absorption at this wavelength. The 8–14- μm region is characterised by many overlapping bands of organic molecules often not readily assignable to specific fundamentals. Conversely, the patterns are complex enough to characterise molecules as a whole. If the identification

of the fundamentals suggested above proves correct, bands in the 8–14- μm region could provide more detailed information on the particle composition.

The carbonaceous material would probably be strongly absorbing in the visible or ultraviolet. Small particles of this material would be heated efficiently, possibly accounting for the high temperatures required to account for the emission bands in a nebula like NGC7027 (ref. 2). In regions where different solids occur (as distinct particles), temperatures would depend on the individual particle compositions through the emissivities and absorptivities. Small carbonaceous particles with strong ultraviolet absorption would reach elevated temperatures if their infrared emissivity is not too high. This could explain why the unknown features always seem to be in emission even when the silicates are in absorption.

The material proposed here is related in some ways to the graphite-like material which has long been suspected as a possible grain constituent. Unlike some previous suggestions, the carbonaceous particles would not have to meet stringent requirements on size, shape, or purity, at least to give the degree of agreement with the infrared observations discussed above. There seem to be similarities in the grains and in carbonaceous chondrites¹⁶. Further laboratory and observational work, especially in the 5–14- μm wavelength region, may show whether a second meteoritic material, the organic polymer-like material in carbonaceous chondrites, also has an analogue in the interstellar dust.

I thank J. Bregman for pointing out that the methyl group has an 11.3- μm absorption and for useful comments.

R. F. KNACKE

Department of Earth and Space Sciences,
State University of New York,
Stony Brook, New York 11794

Received 3 June; accepted 12 July 1977.

- 1 Gillett, F. C., Forrest, W. J. & Merrill, K. M. *Astrophys. J.* **183**, 87 (1973).
- 2 Grasdalen, G. & Joyce, R. R. *Astrophys. J. Lett.* **205**, L11 (1976).
- 3 Merrill, K. M., Soifer, B. T. & Russell, R. W. *Astrophys. J. Lett.* **200**, L37 (1975).
- 4 Bregman, J. D. & Rank, D. M. *Astrophys. J. Lett.* **195**, L125 (1975).
- 5 Russell, R. W., Soifer, B. T. & Willmer, S. P. *NASA TMX-73*, **190**, 58 (1977).
- 6 Russell, R. W., Soifer, B. T. & Merrill, K. M. *Astrophys. J.* **213**, 66 (1977).
- 7 Ridgway, S. T., Hall, D. N. B., Kleinmann, S. G., Weinberger, D. A. & Wojslaw, R. S. *Nature* **264**, 345 (1976).
- 8 Salpeter, E. E. *Astrophys. J.* **193**, 579 (1974).
- 9 Czyzak, S. J. & Santiago, J. J. *Astrophys. Space Sci.* **23**, 443 (1973).

- ¹⁰ Nagy, B. *Carbonaceous Meteorites* (Elsevier, Amsterdam, 1975).
¹¹ Woeller, F. & Ponnampetuma, C. *Icarus* 10, 386 (1969).
¹² Khare, B. N. & Sagan, C. *Icarus* 20, 311 (1973).
¹³ Scattergood, T., Lesser, P. & Owen, T. *Icarus* 24, 465 (1975).
¹⁴ Herbig, G. H. *Mem. Soc. Roy. Sci. Liège Ser. 5*, 19, 13 (1970).
¹⁵ Knacke, R. F. *Nature* 217, 44 (1968).
¹⁶ Zaikowski, A. & Knacke, R. F. *Astrophys. & Space Sci.* 37, 3 (1975).
¹⁷ Gillett, F. C., Kleinmann, D. E., Wright, E. L. & Capps, R. W. *Astrophys. J. Lett.* 198, L65 (1975).
¹⁸ Meinschein, W. G., Nagy, B. & Henessy, D. J. *Ann. N.Y. Acad. Sci.* 108, 553 (1963).

Lateral variation of phenocryst assemblages in volcanic rocks of the Japanese islands

To understand the origin of calc-alkaline rocks and island arc volcanism, knowledge of the lateral variation of the petrological characters of volcanic rocks is essential. The lateral variation of chemical compositions of volcanic rocks has been investigated by several authors¹⁻⁴ and correlated to the depth of the Benioff zone. The east volcanic zone of Japanese Islands, which contains about 140 discrete Quaternary volcanoes, is the most extensively studied volcanic zone in the world. I have examined the petrological data of these volcanoes, particularly with respect to the phenocryst assemblages in volcanic rocks and their changes during the history of respective volcanoes. The references have been mostly taken from 200 papers compiled by Isshiki *et al.*⁵

The volcanoes are classified into three groups on the basis of the presence or absence of hornblende and biotite phenocrysts in essential materials: (1) those without biotite and hornblende phenocrysts in any of the lavas or pyroclastics; (2) those with hornblende phenocrysts but without biotite phenocryst at least in one lava flow or pyroclastic rock; and (3) those with biotite phenocrysts at least in one lava or pyroclastic rock. The distribution of the volcanoes of these three groups is shown in Fig. 1.

Volcanic rocks containing biotite phenocrysts were erupted only in the western part of the volcanic zone, whereas those which do not contain any hydrous mineral phenocrysts were erupted in the eastern part of the volcanic zone. Volcanoes in a belt situated between the above two belts erupted rocks containing only hornblende as hydrous mineral phenocryst.

Phenocrysts such as pyroxene, hornblende, and biotite are considered to be precipitated in a magma reservoir or

during the ascent of magma. The lateral variation outlined above may be due to the lateral change in physicochemical conditions of magma reservoirs across the volcanic zone. It may also be due to a lateral variation in the chemical composition of the original magmas, for example, their alkali contents—especially the K₂O contents³ and possibly to the variation of H₂O contents in the original magma which may increase westwards across the volcanic zone.

I thank Professor I. Kushiro and Dr R. H. Grapes for their comments and criticisms.

M. SAKUYAMA

*Geological Institute,
University of Tokyo,
Tokyo, Japan*

Received 31 May; accepted 5 July 1977.

- ¹ Kuno, H. *Bull. Volcanol.* 29, 195 (1966).
² Kuno, H. *Bull. Volcanol.* 32, 141 (1968).
³ Hatherton, T. & Dickinson, W. R. *J. geophys. Res.* 74, 5301 (1969).
⁴ Sugimura, A. *Bull. Volcanol. Soc. Jap. 2nd Ser.* 4, 77 (1959).
⁵ Isshiki, N., Matsui, K. & Ono, K. *Geol. Surv. Jap.* (1968).

Rare earth element mobility and geochemical characterisation of spilite rocks

HELLMAN & Henderson¹ have demonstrated the enrichment of the rare earth elements (REE) in the lower spilite portion of the Bhoiwada lavic profile relative to the fresh tholeiitic flow top. The remarkable feature of the REE distribution in the spilites was the absolute enrichment of all the elements and not only the light REE, as occasionally seen in oceanic basalts due to submarine weathering². Thus the enhanced spilite REE distribution resembles either alkali basalt or tholeiitic differentiation products rather than tholeiitic basalt. As stated¹, the high degree of REE mobility suggests that these elements must be used with caution in determining the initial magma type or volcanic environment of ancient spilite rocks.

At face value the REE distribution in the spilites is very similar to that exhibited by the more alkaline units of the Deccan basalts^{3,4}. This feature suggests the possibility that the Bhoiwada profile may not be a single flow unit at all and the fresh tholeiitic 'top' may be a different unit to the more 'alkaline' spilite base. Although the section is described as gradational^{5,6}, there are primary textural differences (pillowed/massive; ophitic nature; vesicularity and so on) between tholeiite and spilite, and the contact seems sharp (see photomicrographs and photograph in refs 5 and 6). If the sub-aqueous (flow 'base') sub-aerial (flow 'top') origin postulated for the flow⁵ is correct, then it lacks some of the features described for such lava flows^{7,8} and does not adequately explain why spilite should have stopped abruptly at the water-air interface. Also the considerable REE distribution gap (Fig. 2a of ref. 1) between tholeiite and spilite might lend support to the differences in the two zones, especially as there is no apparent gradational increase in normalised REE patterns with progressive spilite. This is particularly noticeable in the sudden jump of the La/Yb ratio¹ from tholeiite to spilite and also the chemical similarity between the spilite and the (separate) basal pillow lava unit.

Irrespective of whether the profile represents a single unit or not, however, independent criteria are required to characterise the spilite and compare it with the fresh tholeiite. A variety of geochemical (and mineralogical) parameters, which will give the same characterisation, are necessary to determine the nature of ancient altered volcanics. Vallance^{6,9} has advocated the use of relict pyroxenes to determine the magma type of spilite rocks. In the case considered here, the pyroxenes in both tholeiite and spilite are low-Ca augites of tholeiitic character and are similar in composition⁶. Apart from the REE, other relatively 'immobile elements', such as Ti, P, Zr, Y and Nb, have been used to determine magma type^{10,11} and again demonstrates the tholeiitic nature of

Fig. 1 Distribution of Quaternary volcanoes in Hokkaido and northern Honshu. ●, Volcanoes without biotite and hornblende phenocrysts in any of the lavas or pyroclastics; ○ those with hornblende phenocrysts but without biotite phenocryst at least in one lava flow or pyroclastics; open circles represent those with biotite phenocrysts at least in one lava or pyroclastics.

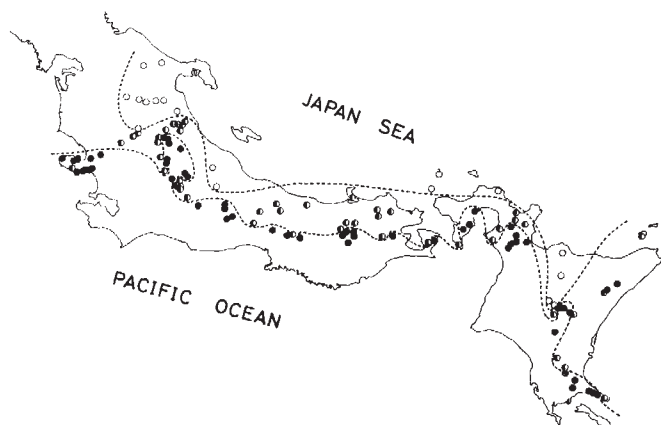


Table 1 Variation in chemical parameters from the Bhoiwada profile^{1,6}

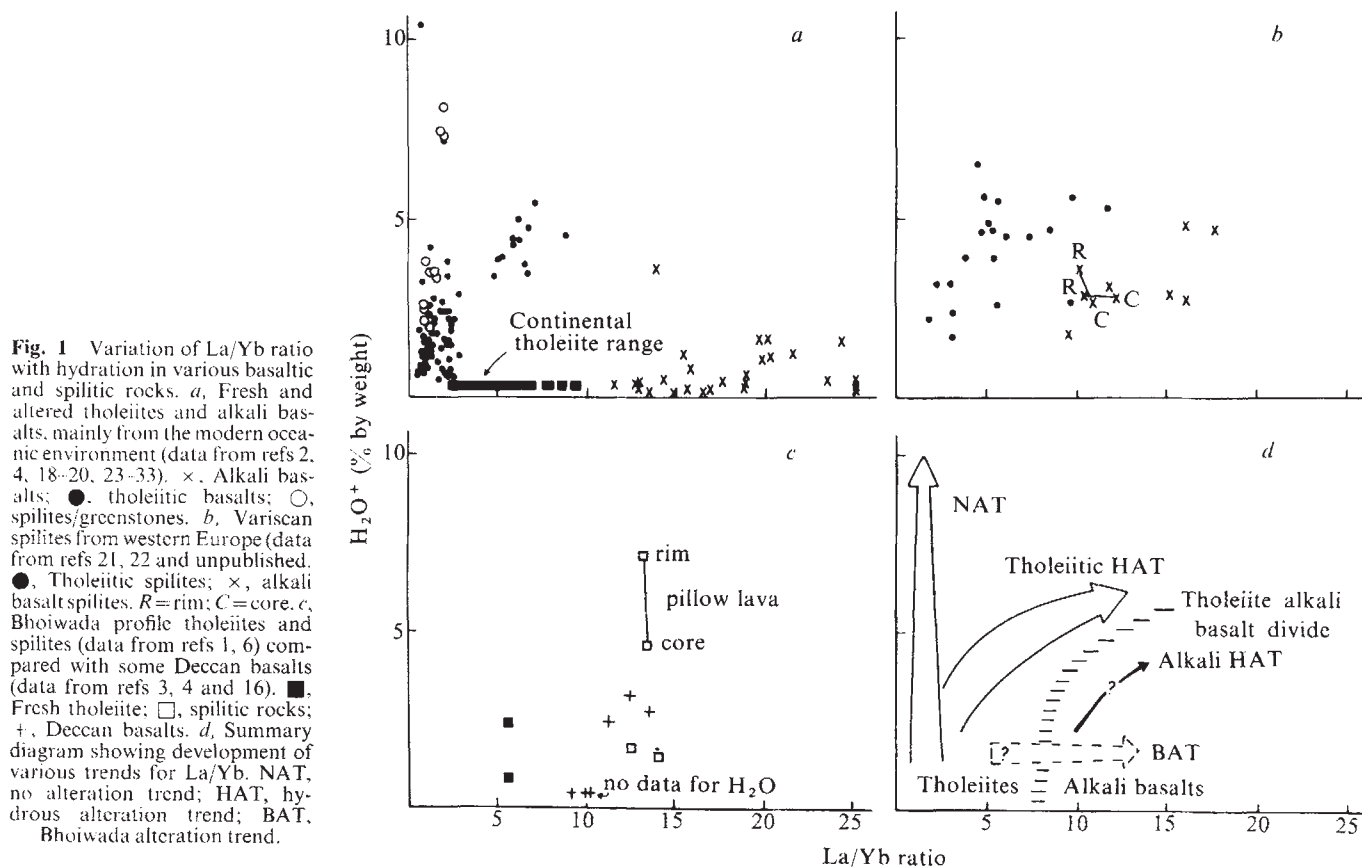
Samples	Tholeiite		Spilitite		Basal pillow unit	
	I	II	III	IV	V (core)	V (rim)
Whole rock						
Zr/TiO ₂	0.010	0.015	0.012	0.010	0.013	0.011
Zr/P ₂ O ₅	0.058	0.115	0.120	0.100	0.026	0.025
La/Yb	5.4	5.5	14.0	12.4	13.2	13.1
FeO*/MgO	2.15	1.99	1.78	1.75	2.46	1.55
Fe ₂ O ₃ /FeO	0.27	0.59	0.46	0.65	0.56	0.85
H ₂ O ⁺	0.87	2.46	1.47	1.71	4.61	7.14
Pyroxene fractions (Σ Fe/Mg, at. %)						
Light	0.42	0.38	0.39	0.37		
Medium	—	0.77	—	—		
Heavy	0.65	1.07	0.66	0.62		

the spilite on the basis of the TiO₂-Zr/P₂O₅ diagram (Fig. 3 of ref. 12). But, there are differences (as well as clear similarities) between the rock units in terms of immobile element ratios (Table 1). Note in particular the more fractionated character of sample II⁶ (sample 2 of ref. 1) relative to sample I (sample 1) as shown by the higher Zr/TiO₂ ratio (see refs 13 and 14 for usage of this ratio). This is substantiated by the higher Σ Fe/Mg ratio of the separated heavy pyroxene fraction from sample II, although not apparently by the lighter fraction (Table 1). The spilites have similar Zr/TiO₂ ratios to the freshest tholeiite (sample I) and thus exhibit a similar degree of basaltic differentiation.

This example from the Bhoiwada profile illustrates the use of variable discriminatory parameters applied to spilitic rocks (compare ref. 13), but is at variance with the REE 'alkaline' designation. But, before any spilite with apparent 'alkali basalt' characteristics is dismissed as an REE-enriched tholeiite, we need first, to check the designation against other geochemical and mineralogical criteria (as above) and second, to test the degree of REE mobility against some alteration parameter, such as H₂O⁺, in each situation. With pillow lavas it is also customary to compare the rim and core portions to determine the zone of possible enrichment.

I now briefly examine the effect of progressive hydration (as H₂O⁺) on the REE in relatively modern basalts and compare the distribution and fractionation (as the La/Yb ratio) with spilites from the Variscan of western Europe. Within this framework the Bhoiwada basalt-spilite fractionation pattern can be compared. It must be emphasised that H₂O⁺ is only one of several possible alteration parameters¹⁵, although in view of the development of hydrous low-grade assemblages in spilites and also hydrated glass in modern sea-floor basalts, it seems the most appropriate (compare ref. 13).

Figure 1a is a plot of relatively recent tholeiitic and alkaline basalts mainly from the oceanic environment and shows their separation on the basis of the La/Yb ratio (representing light to heavy REE fractionation). The relative position of continental tholeiites is indicated schematically, due to the general lack of H₂O⁺ determinations and is based on data from various localities^{4,16,17}. The common La/Yb range for continental tholeiites is about 2-7 (with a few values up to ~10), whereas continental and oceanic alkali basalts show a much wider range with La/Yb ~11-40 (Fig. 1a and ref. 17). Associated oceanic island tholeiites and alkali basalts from Hawaii¹⁸ show La/Yb ranges of 3.9-6.8



and 7.8–15.6 respectively and coupled with the above data provide an approximate magma type divide at La/Yb of ~ 7 –8.

The ocean floor/ridge tholeiites in Fig. 1a exhibit two trends with increasing hydration—(1) increasing La/Yb in samples with $H_2O^+ > 3\%$ by weight and (2), no change in La/Yb, irrespective of H_2O^+ content, even up to very high values (10% by weight). Both of these features are seen in basalts that have suffered low-temperature alteration with palagonitisation of glass and developed smectitic clays, carbonate, Fe oxides and rare zeolites. Also higher temperature alteration, exhibited by recent ocean floor rocks and generally termed greenstones or spilites (for example refs 19 and 20), show no significant change in the La/Yb ratio with progressive hydration. Unfortunately there is a lack of analyses of sufficiently altered alkali basalts to show any similar trends, although within any one volcanic province the variation is minimal (up to 2% H_2O^+ by weight) in rocks of similar basaltic composition.

Figure 1b shows the variation of the La/Yb ratio with hydration in various Upper Palaeozoic spilites from western Europe. This includes REE data from north-western Germany^{21,22}, south western England, Brittany and southern Portugal (unpublished data by PAF). The south-western England data includes both intrusive sills and pillow lavas that have been designated as tholeiites or alkali basalts on the basis of the distribution of other 'immobile' elements (refs 11, 13 and Table 2). Note that the La/Yb ratio increases with progressive hydration

enrichment²⁴, but not to the degree as illustrated by the Bhoiwada spilite. As seen in Table 2, the pillow rims are enriched relative to their cores, with the enrichment in both cases being almost entirely due to Ce and to a lesser extent Sm.

Figure 1c shows the Bhoiwada lava data^{1,6} and various Deccan basalts^{3,4,16}, which characterised by high La/Yb ratios suggest an 'alkaline' character for these particular samples. Compared with the alteration trends derived from Fig. 1a and b, the development of the Bhoiwada spilite is unusual in showing a marked La/Yb enrichment without any attendant H_2O^+ increase. Also, if the basal pillow lava unit was originally tholeiitic, the whole pillow has been REE enriched such that the rim and core have developed similar La/Yb ratios. The pillow lava, due to its high H_2O^+ content, could fall on the end of the altered basalt/spilite trend of Fig. 1a and b. Certainly the form of variation exhibited by the Bhoiwada profile is different from that seen elsewhere and may define another (abnormal?) trend. It is difficult to see what this difference may be due to, as the Bhoiwada alteration products (chlorite, albite, prehnite, laumontite⁵) are not markedly different from the mineralogy of some ocean floor spilites. Adsorption of La (and other REE?) by layer silicates has been suggested¹ as a factor in determining REE distribution patterns, although equally chloritic spilites (or clay-rich altered basalts) do not show such a high degree of enrichment in the marine environment.

In conclusion, it seems that the REE can be fractionated during alteration, although the trends of alteration in such cases can still

Table 2 Variation of immobile element ratios and chemical alteration criteria in associated portions of massive and pillowed spilites from south-western England

Sample no. Portion	Massive sill		Pillow lavas			
	CG12 Central	CG16 Margin	PP7 Core	PP7 Rim	PP10 Core	PP10 Rim
H ₂ O ⁺ (weight %)	3.96	5.53	2.77	2.88	2.67	3.56
Fe ₂ O ₃ /FeO	0.10	0.12	0.37	0.34	0.23	0.63
Σ REE (p.p.m.)	27.19	24.83	100.97	113.07	102.13	120.44
La/Sm	2.2	2.8	5.2	3.6	4.8	3.5
La/Yb	5.3	5.6	12.2	10.4	10.9	10.1
Zr/TiO ₂	0.005	0.007	0.006	0.006	0.006	0.005
Zr/P ₂ O ₅	0.086	0.076	0.053	0.060	0.092	0.055
Nb/Y	0.40	0.40	1.14	1.15	1.00	1.50
Immobile element classification	Tholeiitic basalt		Alkali basalt		Alkali basalt	
Clinopyroxene type	Common augite + rare pigeonite exsolution lamellae		(no pyroxene relicts)			

(especially in samples with $H_2O^+ > 3\%$ by weight) in a similar manner to one of the trends exhibited by the altered oceanic floor tholeiites of Fig. 1a. Although the La/Yb ratio in the altered tholeiites increases towards more alkaline values (~ 10) this only takes place at a relatively high level of alteration (5–6% H_2O^+ by weight) and would only be confused with alkali basalts if their La/Yb ratio remained constant at the same level of alteration. The alkali basalt designated spilites (largely from south-western England) have typical alkaline La/Yb ratios and are separate, in terms of alteration trends, from the tholeiitic spilites. In the alkali basalt pillow lavas the La/Yb ratio marginally decreases from core to rim (Table 2). de Paape *et al.*²³ have also demonstrated the lack of variation in pillow lava segments with core and glassy crust La/Yb ratios being essentially the same, although the H_2O^+ content is considerably different.

Together with variation in the ocean floor basalts, the plotted spilite data suggests variation of the La/Yb ratio with alteration, although the trends of variation seem to still allow spilitic tholeiites to be distinguished from spilitic alkali basalts. The total content of REE in spilites derived from known basaltic parents shows a small

be used to distinguish tholeiites from alkali basalts. To determine if alteration has changed the La/Yb ratio of a basaltic suite it is necessary to use a plot such as Fig. 1, before this ratio is applied to petrogenetic modelling. The designation of spilitic rocks to one magmatic series or another is important in elucidating the nature of ancient volcanism and must be determined using a variety of geochemical 'immobile' parameters. Figure 1d summarises the alteration trends and marks a boundary between altered tholeiites and alkali basalts. This division remains highly tentative, however, until alkali basalts, hydrated to the same level as some tholeiites, have been analysed to check if any change in their La/Yb ratio takes place. If not, then alkali basalts with La/Yb ratios of ~ 10 –12 will overlap with altered tholeiites containing 5–6% H_2O^+ by weight. Both the no-alteration trend (NAT) and the hydrous alteration trend (HAT) are characterised by greenschist facies spilites and very low-grade alteration (submarine weathering) of basalts. What factors determine which of these two main trends develop is unclear (longevity of burial; sedimentation rate; nature of lava units?) and in particular why do modern spilites follow NAT and ancient spilites follow HAT? The Bhoiwada trend

(BAT) is also shown, although further studies are required to substantiate this type of alteration.

I thank Dr J. A. Winchester for helpful comments.

P. A. FLOYD

Department of Geology,
University of Keele,
Keele, Staffordshire, UK

Received 13 June; accepted 5 July 1977.

- ¹ Hellman, P. L. & Henderson, P. *Nature* **267**, 38–40 (1977).
- ² Frey, F. A., Bryan, W. B. & Thompson, G. *J. geophys. Res.* **79**, 5507–5527 (1974).
- ³ Alexander, P. O. & Gibson, I. L. *Lithos* **10**, 143–147 (1977).
- ⁴ Frey, F. A., Haskin, M. A., Poetz, J. A. & Haskin, L. A. *J. geophys. Res.* **73**, 6085–6089 (1968).
- ⁵ Sukheswala, R. N. in *Spilites and Spilitic rocks* (eds Amstutz, G. C. et al.) 229–250 (Springer, Heidelberg, 1974).
- ⁶ Vallance, T. G. *J. Petrology* **15**, 79–96 (1974).
- ⁷ Jones, J. G. & Nelson, P. H. *Geol. Mag.* **107**, 13–19 (1970).
- ⁸ Furnes, H. & Sturt, B. A. *J. Geol.* **84**, 439–453 (1976).
- ⁹ Vallance, T. G. in *Spilites and Spilitic Rocks* (eds Amstutz, G. C. et al.) 59–68 (Springer, Heidelberg, 1974).
- ¹⁰ Pearce, J. A. & Cann, J. R. *Earth planet. Sci. Lett.* **19**, 290–300 (1973).
- ¹¹ Floyd, P. A. & Winchester, J. A. *Earth planet. Sci. Lett.* **27**, 211–218 (1975).
- ¹² Winchester, J. A. & Floyd, P. A. *Earth planet. Sci. Lett.* **28**, 459–469 (1976).
- ¹³ Floyd, P. A. *J. Petrology* **17**, 522–545 (1976).
- ¹⁴ Winchester, J. A. & Floyd, P. A. *Chem. Geol.* (in the press).
- ¹⁵ Hart, S. R., Erlank, A. J. & Kable, E. J. *D. Contrib. Mineral. Petrol.* **44**, 219–230 (1974).
- ¹⁶ Balashov, Yu. A. & Nesterenko, G. V. *Geochem. Intern.* **3**, 672–679 (1966).
- ¹⁷ Herrmann, A. G. *Contrib. Mineral. Petrol.* **17**, 275–314 (1968).
- ¹⁸ Schilling, J. G. & Winchester, J. W. *Contrib. Mineral. Petrol.* **23**, 27–37 (1969).
- ¹⁹ Melson, W. G., Thompson, G. & van Andel, T. H. *J. geophys. Res.* **73**, 5925–5941 (1968).
- ²⁰ Cann, J. R. *J. Petrology* **10**, 1–19 (1969).
- ²¹ Herrmann, A. G. & Wedepohl, K. A. *Contrib. Mineral. Petrol.* **29**, 255–274 (1970).
- ²² Herrmann, A. G., Potts, M. J. & Knake, D. *Contrib. Mineral. Petrol.* **44**, 1–16 (1974).
- ²³ de Paeppe, P., Klerkx, J., Hertogen, J. & Plinke, P. *Earth planet. Sci. Lett.* **22**, 347–354 (1974).
- ²⁴ Nicholls, G. D. & Islam, M. R. *Phil. Trans. R. Soc.* **268**, 469–486 (1971).
- ²⁵ Masuda, A. & Nagasawa, S. *Geochem. J.* **9**, 227–233 (1975).
- ²⁶ Blanchard, D. P. et al. *J. geophys. Res.* **81**, 4231–4246 (1976).
- ²⁷ Kay, R., Hubbard, N. J. & Gast, P. W. *J. geophys. Res.* **75**, 1585–1614 (1970).
- ²⁸ Goldich, S. S., Treves, S. B., Suhr, N. H. & Stuckless, J. S. *J. geol.* **83**, 415–435 (1975).
- ²⁹ Flower, M. F. J. *Contrib. Mineral. Petrol.* **31**, 335–346 (1971).
- ³⁰ Price, R. C. & Taylor, S. R. *Contrib. Mineral. Petrol.* **40**, 195–206 (1973).
- ³¹ Tanaka, T. & Sugisaki, R. *J. Petrology* **14**, 489–508 (1973).
- ³² Engel, A. E. J., Engel, C. G. & Havens, R. G. *Bull. geol. Soc. Am.* **76**, 719–734 (1965).
- ³³ Frey, F. A. *Earth planet. Sci. Lett.* **7**, 351–360 (1970).

Transuranic depositional history in South Greenland firn layers

THE surface layers of the Greenland ice sheet have preserved a continuous and detailed record of atmospheric fallout of transuranic nuclides from weapons tests over the past 30 yr. The ages of the glacial strata were determined by ^{210}Pb geochronologies. We found that the influence of remobilised soil debris with their sorbed transuranics does not seem to be important, and that fallout maxima occurred in the 1950s and 1960s for $^{239+240}\text{Pu}$ and in 1965–66 for ^{238}Pu . This work may be extended by using alpine glaciers in mid-latitudes for a reconstruction of fallout patterns over the past three decades and for an evaluation of present day dispersions of these nuclides as a consequence of atmospheric fallout and other possible entries through the nuclear fuel cycle.

The observed or measured stratigraphic layers found in glaciers of the polar regions are very attractive for studies of the various species of atmospheric fallout including pollutants. Although glaciers are found at nearly every latitude of the Earth, it is the inland areas of the Greenland and Antarctica ice sheets that provide the most reliable stratigraphical relations¹. This is because summer melting is negligible or non-existent, and orderly sequential buildup is not destroyed by percolation. The snow or firn and the dissolved and particulate substances are essentially immobile following deposition². A number of geochronologies, both radiometric and stratigraphic, allow the introduction of time frames into the layered systems; in many instances, the season of the year, as well as the year itself, can be identified³. The fluxes of some metals from the atmosphere to the glaciers over the past centuries and millenia have identified both the anthropogenic and natural contributions^{4–8}. The fallout of nuclear debris from weapons testing has been observed in both the Arctic and Antarctic glaciers through gross β , ^{90}Sr and ^{137}Cs measurements^{9–13}. With increasing concerns about the dispersion of transuranics in our environment, it was of interest to develop the

pollution history of Pu and Am isotopes from a Greenland ice sheet location.

During the 1975 field operations of the Greenland Ice Sheet Program (GISP) a 30-m deep, 7.6-cm diameter firn core was augered at a location designated 'South Dome' Greenland ($63^{\circ}31.6'\text{N}$, $44^{\circ}34.5'\text{W}$). The June 1975 surface serves as the zero depth horizon. A lightweight mechanical drill was used¹⁴. Both the core and the chips formed during drilling were collected in the same polyethylene bag from each approximate 1-m run of the drill. After being transported frozen to the US, the 4–9-kg samples (dependent on the general increasing density with depth) were transferred to 10-l polyethylene containers, rapidly melted in a microwave oven and acidified to a pH of 1 with HNO_3 .

The chemical analyses were performed on the residues obtained by the evaporations of the 4–9-l of melt water. Pu analyses were carried out¹⁵. Following melting, the waters were acidified with HNO_3 . Later, spikes of Pb carrier and the tracers ^{242}Pu and ^{243}Am were added. The Am contents were determined by a modification of an unpublished method¹⁶. ^{210}Pb assays were made by a routine method developed at the Scripps Institution of Oceanography¹⁷.

The sedimentation rate over the 30-m profile averaged 1 m yr^{-1} for the gross glacial material or 0.6 m yr^{-1} of water equivalent, after density corrections on the basis of ^{210}Pb chronology (Fig. 1; ref. 18). This value compares well with the accumulation rate determined on the basis of firn stratigraphy and oxygen isotopes (Dansgaard et al., personal communication).

Table 1 $^{241}\text{Pu}/^{239+240}\text{Pu}$ ratios, computed at various depths in the Greenland Glacier for the year 1962

Depth (m) water equivalent	Time (yr)	$^{241}/^{239+240}\text{Pu}^*$
0–0.58	0–1.0	31.4
0.58–1.06	1.0–1.7	35.3
1.06–1.45	1.7–2.4	45.0
1.45–1.83	2.4–3.0	21.5
1.65–2.14	3.0–3.5	26.9
2.14–5.28	3.5–4.2	11.1
2.58–3.10	4.2–5.1	15.6
3.10–4.14	5.1–6.7	13.2
4.14–5.16	6.7–8.5	32.0
5.16–5.73	8.5–9.4	14.1
5.73–6.37	9.4–10.4	16.0
6.37–7.03	10.4–11.5	17.1
7.03–7.72	11.5–12.0	18.9
7.72–8.46	12.0–13.0	11.5
8.46–9.02	13.0–14.8	16.9
9.02–9.51	14.8–15.6	11.1
9.51–10.3	15.6–16.9	8.6
10.3–10.77	16.9–17.7	11.8
10.77–11.77	17.7–19.3	20.5
11.77–12.3	19.3–20.2	23.2
12.3–12.9	20.2–21.1	21.6
12.9–13.5	21.1–22.1	26.4
13.5–14.1	22.1–23.0	28.6
14.1–15.4	23.0–25.3	24.7
15.4–16.5	25.3–27.1	39.2
17.8–19.2	29.2–31.5	32.8

*For 0–13.9 yr ago, the $^{241}\text{Pu}/^{239+240}\text{Pu}$ are normalised to a parameter of ^{241}Pu in July 1962 at which time the level of ^{241}Am was zero. For times 13.9–22.1 yr ago, a similar normalisation was made to 1954. For earlier times, the ^{241}Pu is calculated assuming ^{241}Am was zero at this time and decay is taken into account.

The Pu and Am isotopic concentrations as a function of time of deposition or of depth are shown in Fig. 1 with the ^{210}Pb concentrations which were used to establish one of the chronologies. Two distinct maxima are observed for $^{239+240}\text{Pu}$ for the periods 1963–65 and 1955–60. A third maximum may exist in the strata deposited between 1946 and 1948, and may reflect the Nagasaki, Hiroshima and/or Almagordo, New Mexico weapon detonations. The ^{238}Pu profile in the glacial deposits is similar to that of the $^{239+240}\text{Pu}$ up to 1966. The input of this isotope from the burn-up of the SNAP-9A device in 1964, is not recorded in the

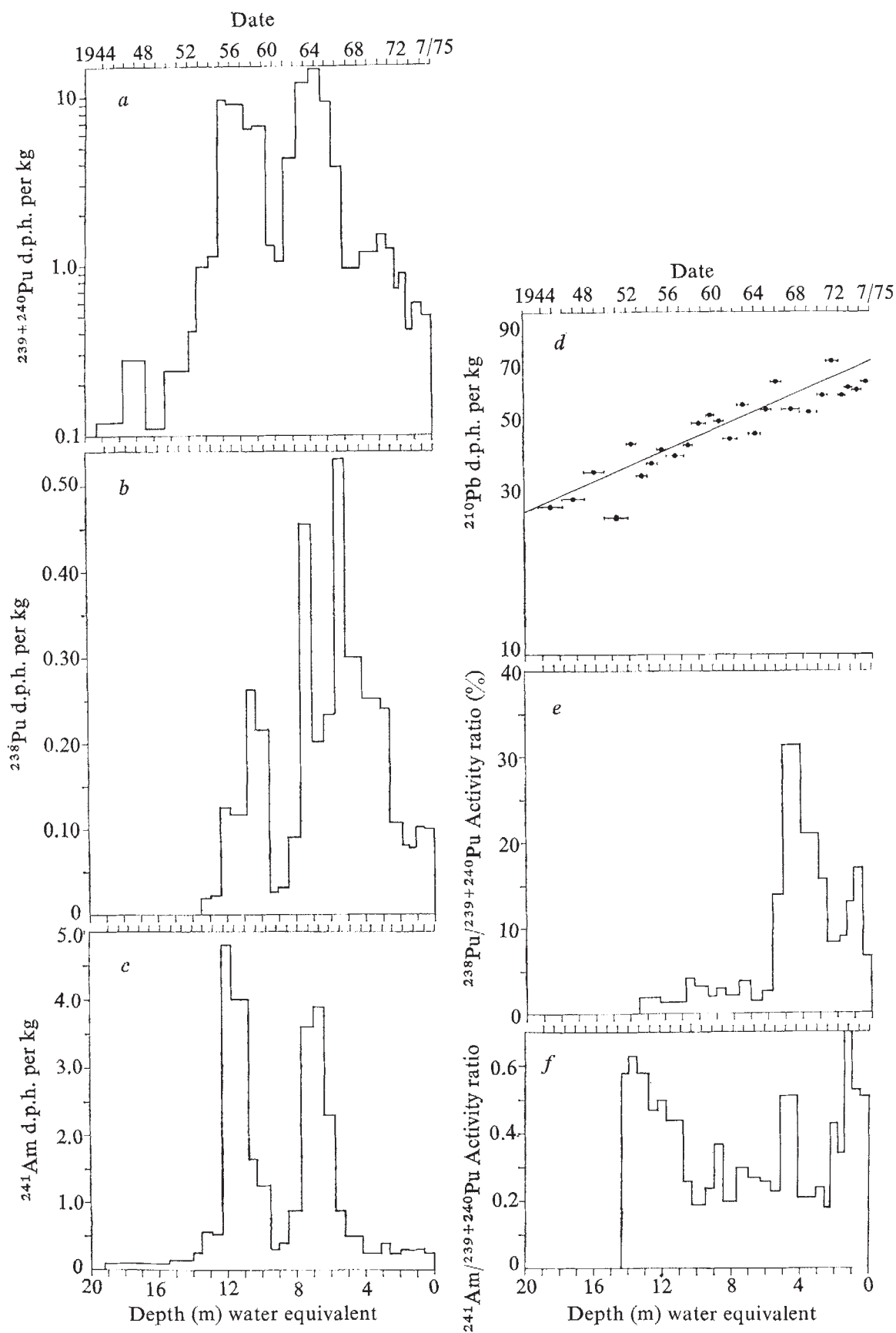


Fig. 1 *a-d*, $^{239} + ^{240}\text{Pu}$, ^{238}Pu , ^{241}Am and ^{210}Pb activities in the Greenland Ice Sheet. *e, f*, The $^{238}\text{Pu}/^{239} + ^{240}\text{Pu}$ and $^{241}\text{Am}/^{239} + ^{240}\text{Pu}$ activity ratios in the Ice Sheet. The timescale is based on the ^{210}Pb geochronology. The counting errors for these nuclides were higher for the surface samples, as a consequence of small sample size, and for the deeper samples, as a consequence of low concentrations of the nuclides. The average counting errors and their ranges (in parentheses) are: $^{239} + ^{240}\text{Pu}$, 7.4% (2.8–15.6); ^{241}Am , 13% (6.2–33); and $^{238}\text{Pu}/^{239} + ^{240}\text{Pu}$, 31% (12.5–53). For ^{210}Pb , the counting errors were 3% or less for all samples.

glacier until 1966. The patterns of ^{241}Am levels are in concord with that of $^{239+240}\text{Pu}$; this nuclide grows in from the decay of its parent ^{241}Pu , produced in weapons testing along with the other Pu isotopes.

The most extensive record of plutonium fallout from the atmosphere as a function of latitude (for samples collected between October 1970 and January 1971) has been carried out by the Health and Safety Laboratory of ERDA in New York¹⁹. Soil samples to a depth of 30 cm, with a surface area of 622 cm² were collected at 65 sites. The assumption was made that the soils were undisturbed and had integrated weapons fallout over the past 25 yr or so. There is, however, strong evidence that soils can be mobilised by winds and waters with a consequential alteration of the fallout record. Coastal sediments often show increasing $^{239+240}\text{Pu}$ fluxes from the 1950s to the present, whereas if stratospheric fallout were the only entry, maxima would be expected in the late 1950s and early 1960s.

The contribution of soil mobilised $^{239+240}\text{Pu}$ to the amount of these isotopes in the Greenland ice sheet can be estimated in the following way. Windom²⁰ has indicated that the fallout rate of particles in a Greenland glacier is 21 mg per cm² per 10³ yr. If we assume a Pu activity of 1.0 d.p.m. per g for atmospheric particles, similar to that observed for northern hemispheric dusts¹⁵, then the remobilised soil debris is contributing 2.1×10^{-5} d.p.m. per cm² per yr. For surface samples of the glacier, there is an accumulation rate of 60 cm, water equivalent per yr, or 0.06 kg per cm² per yr¹. Using a $^{239+240}\text{Pu}$ concentration of 0.01 d.p.m. per kg for the surface strata of the glacier, the total flux of $^{239+240}\text{Pu}$ is of the order of 6×10^{-4} d.p.m. per cm² per yr. Thus, the soil remobilised $^{239+240}\text{Pu}$ may contribute the order of 5% of the total burden of these isotopes in the glacier.

On the basis of the soil record, there has been a cumulative fallout of $^{239+240}\text{Pu}$ for northern latitudes between 60° and 70°N of 1.6 ± 1.0 mCi km⁻² up to 1971. Our integrated value for the ice sheet accumulation through 1975, 0.7 mCi per km², agrees with the previous value.

Through a comparison of transuranic and ^{210}Pb concentrations in a sample collected about 14°N of the one analysed in this work (Camp Century, 77°10'N, 61°08'W; elevation of 1,886 m)⁷, it seems that there may be a uniform fallout of these nuclides over a wide area in Greenland. The Camp Century collection was made in 1965 and the upper layers which were analysed represented snow deposited between the Autumn of 1964 and the Summer of 1965. The concentration of the nuclides in the Camp Century surface layers and 'South Dome' 6 m stratum, corresponding to a time about 1965, are: $^{239+240}\text{Pu}$, 11.3 ± 0.003 and 9.3 d.p.h. kg⁻¹; ^{241}Am , 2.7 ± 0.18 and 2.3 d.p.h. kg⁻¹; $^{238}\text{Pu}/^{239+240}\text{Pu}$, 0.012 ± 0.003 and 0.026 ± 0.005 . The ^{210}Pb of the Camp Century and of a site analysed by Windom²⁰, both decay corrected to the same time, are 96.4 ± 2 and 100 d.p.h. kg⁻¹. These arrangements help dispel the idea that perhaps the results are site specific for any given glacier sample.

Finally, we have examined the ^{241}Am fallout record in relation to that of $^{239+240}\text{Pu}$. It is generally assumed that the ^{241}Am in the environment today arises nearly completely from the decay of ^{241}Pu (half life 14.9 yr) produced in nuclear weapons tests. Although a large number of tests have contributed ^{241}Pu to the environment, we have obtained the ratio $^{241}\text{Pu}/^{239+240}\text{Pu}$ for July 1962 for samples deposited after this time period, for 1954–62 for samples deposited in this interval and for years before 1954. These calculations are described by Livingston *et al.*²¹. These authors indicate that a ratio of 13–14 in fresh debris for the 1961–62 US/USSR tests is representative and clearly our results following the 3.5 most recent years seem to agree with these data. The most recent values of the ratio, ranging from 21.5 to 45, are perhaps explicable by inputs from recent Chinese tests. It should be pointed out that because of small sample sizes, these ratios are less accurate for the near surface and pre-1954 samples.

This research was supported under a Contract with the Energy Research and Development Administration, Environmental Sciences Branch, Division of Biology and Medicine (ERDA E(04-3)-34 P. A. 84) and the National Science Foundation, Division of

Polar Programs (OPP 7609120). Ms Sue Johnson assisted with the transuranic analyses.

MINORU KOIDE
EDWARD D. GOLDBERG

*Scripps Institution of Oceanography,
La Jolla, California 92093*

MICHAEL M. HERRON
CHESTER C. LANGWAY, JR

State University of New York at Buffalo

Received 28 April; accepted 29 June 1977.

- ¹ Langway, C. C., Jr *GSA Special Paper* 125 (1970).
- ² Junge, C. E. *J. geophys. Res.* **65**, 227–237 (1960).
- ³ Dansgaard, W., Johnson, S. J., Clausen, H. B. & Gundestrup, N. *Middeldelevation Grønland*, Bd. 197, Nr. 2, 53 (1973).
- ⁴ Weiss, H., Bertine, K., Koide, M. & Goldberg, E. D. *Geochim. cosmochim. Acta* **39**, 1–10 (1975).
- ⁵ Weiss, H. V., Koide, M. & Goldberg, E. D. *Science* **174**, 692–694 (1971).
- ⁶ Weiss, H. V., Koide, M. & Goldberg, E. D. *Science* **172**, 261–263 (1971).
- ⁷ Murozumi, M., Chow, T. J. & Patterson, C. *Geochim. cosmochim. Acta* **33**, 1247–1294 (1969).
- ⁸ Koide, M. & Goldberg, E. D. *J. geophys. Res.* **76**, 7689–7696 (1971).
- ⁹ Martell, E. A. *Science* **129**, 1197–1206 (1959).
- ¹⁰ Picciotto, E. & Wilgain, S. *J. geophys. Res.* **68**, 5965–5972 (1963).
- ¹¹ Wilgain, S., Picciotto, E. & DeBreuck, W. *J. geophys. Res.* **70**, 6023–6032 (1965).
- ¹² Croxaz, G. *Earth planet. Sci. Lett.* **6**, 6–8 (1969).
- ¹³ Woodward, R. N. *Nature* **204**, 129 (1964).
- ¹⁴ Ruffli, H., Stauffer, B. & Oeschger, H. *Proc. Ice Core Drilling Symp.* (ed. J. Splettstoesser) 139–153 (University of Nebraska, Lincoln, 1974).
- ¹⁵ Koide, M., Griffin, J. J. & Goldberg, E. D. *J. geophys. Res.* **80**, 4153–4162 (1975).
- ¹⁶ Weseman, R. A., Leventhal, L., Lee, K. D. & Major, W. J. paper presented at 18 Conf. *Analyt. Chem. Nuclear Technol.* (Gatlinburg, Tennessee, 1974).
- ¹⁷ Koide, M. & Bruland, K. W. *Anal. chim. Acta* **75**, 1–19 (1975).
- ¹⁸ Goldberg, E. D. in *Radioactive Dating*, 121–131 (International Atomic Energy, 1963).
- ¹⁹ Hardy, E. P., Krey, P. W. & Volchok, H. L. *Nature* **241**, 444–445 (1973).
- ²⁰ Windom, H. L. *Bull. geol. Soc. Am.* **80**, 761–782 (1969).
- ²¹ Livingston, H. D., Schneider, D. L. & Bowen, V. T. *Earth planet. Sci. Lett.* **25**, 361–367 (1975).

Angiosperm fossils from latitude 70°S

WE have found fossil angiosperm leaves more than 1,000 km further south than any other previously reported. Specimens were obtained *in situ* by R. W. B. from tuffs in a volcanic sequence in the eastern Elgar Uplands of northern Alexander Island (Fig. 1). Hitherto in Antarctica, angiosperm macrofossils have been reported only from the northern Antarctic Peninsula area in Tertiary sediments on Seymour Island¹ and in volcanoclastic sequences on King George Island, South Shetland Islands^{2,3}, although angiosperm microfossils, mainly pollen, have been found in erratics of marine sandstone from the McMurdo Sound area⁴. The source area of these latter specimens however, is not known, and it is well known that some pollens, including that of *Nothofagus*, can be transported in detectable quantities over large distances⁵. No macro plant fossils seem to be associated with the McMurdo Sound pollen.

The flora from the Elgar Uplands is poorly preserved but includes the impressions of at least two kinds of leaf (Fig. 2). Numerous carbonised films and moulds, representing accumulation of logs and branches, also occur in north-western Elgar Uplands. The most common kind of leaf (Fig. 2a–c) reaches in excess of 10 cm long and the venation suggest that it had a broad-based ovate outline, although the margin is not clearly delineated on any of the specimens. There is a straight slender mid-vein from which secondary veins branch at angles of 30–40°. On one possible variant (Fig. 2d) this angle of secondary origin is 50° or more. Small fragments of the leaves indicate that each vein terminates in a serration at the leaf margin (Fig. 2a and b). Similar leaf characteristics are commonly observed on specimens from the South Shetland Islands, some of which have been illustrated as *Nothofagus* or '*Fagus*'⁶. Although the present examples are considerably larger than modern species of *Nothofagus* occurring in Patagonia, they are comparable in size with others from New Guinea, New South Wales and the Queensland rain forest. A second kind of leaf (Fig. 2f) is much broader than the first, has an entire margin and an obtusely pointed apex. The mid-vein is slightly sinuous and gives off thin and curved secondary veins at irregular

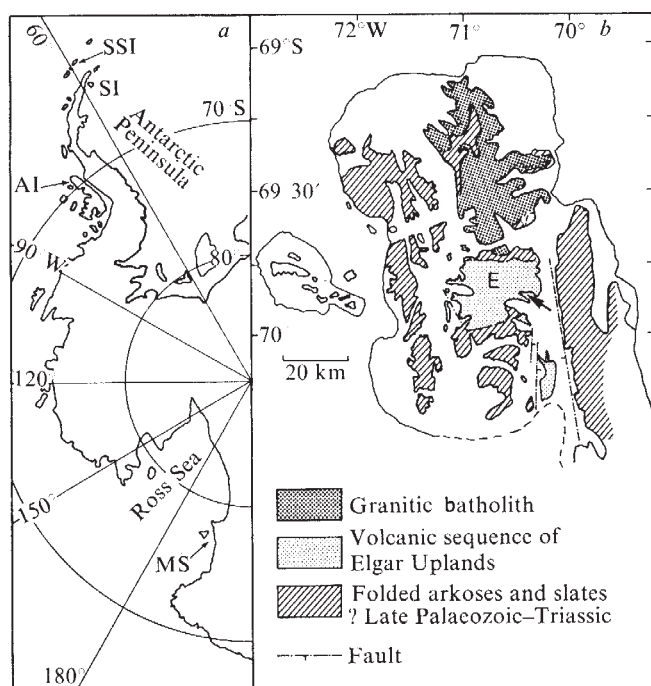


Fig. 1 Maps of Antarctic fossil angiosperm localities. *a*, Lesser Antarctica: AI, Alexander Island; MS, McMurdo Sound; SI, Seymour Island; SSI South Shetland Islands. *b*, Geological map of northern Alexander Island, showing the newly discovered fossil leaf locality (arrow). E, Elgar Uplands.

intervals. The secondary veins seem to have stout triangular bases where they join the mid-vein, but it is possible that this is an accident of preservation rather than an original feature. A fragment of leaf base (Fig. 2*e*) shows irregular venation which suggests closer affinities to the second kind of leaf than the first.

The plant-bearing tuffs occur in a sequence of tuffs, lavas and agglomerates, more than 500 m thick⁷ which crops out over most of Elgar Uplands. The lavas are dominantly basaltic with phenocrysts of zoned labradorite, augite and hypersthene. Only one andesite has been recorded but alteration may preclude their petrological determination. Hornblende together with labradorite are common constituents in the tuffs. All the rocks are extensively altered with abundant calcite, epidote, chlorite and pyrite, and there is widespread silification. A much later stage of volcanism in the area is probably represented by isolated outcrops of fresh vesicular breccias, palagonite breccia and a vesicular olivine basalt plug. These are closely comparable with late Cainozoic volcanic rocks described from Beethoven Peninsula, Alexander Island⁸.

Beneath the volcanic sequences there is a thick succession of deformed sedimentary rocks, assigned to the late Palaeozoic on the basis of fossil spores⁹ and general similarities to comparable rocks elsewhere in the Antarctic Peninsula¹⁰. A late Triassic marine fauna however, has recently been obtained from the (?) upper part of this sequence¹¹.

Field relationships thus set only broad age limits on the plant-bearing tuffs and their precise age is difficult to assess. A K/Ar date of 70 Myr was obtained from a tuff in the lower part of a supposedly related sequence in the nearby Colbert Mountains¹² and this suggests a post-Cretaceous age for much of the volcanic successions. The leaves are too poorly preserved to be identified with certainty but they show some resemblance to those in the South Shetland Islands. Few published accounts of these are available, although one flora from King George Island was assigned to the Miocene on palaeobotanical evidence³. Associated lavas

have now been dated at about 60 Myr (R. J. Pankhurst, personal communication).

These fossils indicate that at some stage in the Tertiary, Alexander Island was colonised by arborescent angiosperms, and their proven *in situ* occurrence is extended 1,000 km south of the northern Antarctic Peninsula area. It is unlikely that Antarctica has moved much since 40 Myr ago¹³ and therefore they point further to a considerably warmer climate than now at approximately the same latitude. The large size of some leaves (>10 cm) could be interpreted to favour the existence of a warm-temperate to subtropical climate¹⁴, but it is unlikely that the present flora is indicative of more than ordinary to warm-temperate conditions.

Evidence has been cited for early and late Cainozoic glaciation in Antarctica, but a review of the data¹⁵ suggests that full-bodied ice sheets developed first in Greater Antarctica sometime after the Lower Miocene (25 Myr ago) and at a later date in Lesser Antarctica. This is supported by fossil evidence from the Ross Sea¹⁶ where microfloral remains in Oligocene glacial deposits are interpreted as representing cool-temperate vegetation, which was finally eliminated in the Miocene.

Although the fossil angiosperm leaves described in this paper are undoubtedly the southernmost yet known, the occurrence of degraded plant tissue, cuticle and vascular fragments, in the Oligocene deposits in the Ross Sea, is thought to indicate a nearby vegetation source in that area¹⁶. Knowledge of the area suggests that, if the source rocks are still preserved, they are hidden by ice.

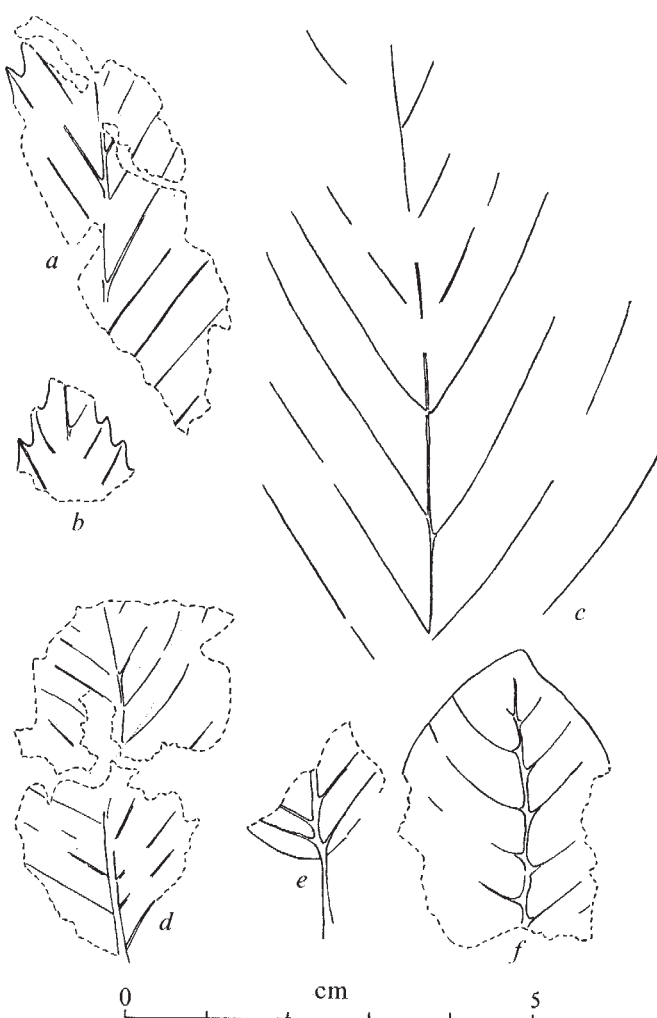


Fig. 2 Camera lucida sketches of fossil angiosperm leaves from Elgar Uplands, Alexander Island.

The fossils are housed in the collections of the British Antarctic Survey at Cambridge. We thank Dr Leo J. Hickey for helpful discussion. The radiometric age date from the South Shetland Islands was determined by Dr R. J. Pankhurst from specimens collected by Dr S. D. Weaver.

M. R. A. THOMSON
R. W. BURN

British Antarctic Survey,
Madingley Road,
Cambridge, UK

Received 26 May; accepted 22 July 1977.

- ¹ Dusén, P. *Wiss. Ergebn. schwed. Südpolarexped.* 3, Lief 3 (1908).
- ² Barton, C. M. in *Antarctic Geology* (ed. Adie, R. J.) 603–8 (North-Holland, Amsterdam, 1964).
- ³ Orlando, H. A. in *Antarctic Geology* (ed. Adie, R. J.) 629–36 (North-Holland, Amsterdam, 1964).
- ⁴ Cranwell, L. M., Harrington, H. J. & Speden, I. G. *Nature* 186, 700–702 (1960).
- ⁵ Darlington, P. J., Jr *Biogeography of the Southern End of the World* (Harvard University, Cambridge, Massachusetts, 1965).
- ⁶ Adie, R. J. in *Antarctic Research* (ed. Adie, R. J.) 118–62 (Butterworth, London, 1964).
- ⁷ Bell, C. M. *Br. Antarct. Surv. Bull.* No. 39, 36–44 (1974).
- ⁸ Bell, C. M. *Br. Antarct. Surv. Bull.* No. 32, 75–83 (1973).
- ⁹ Griukurov, G. E. & Dibner, A. F. *Dokl. Akad. Nauk. S.S.S.R., Geology* 179, 410–412 (1968).
- ¹⁰ Griukurov, G. E. in *Antarktika. Mezhdunarodnaya komissiya po izucheniyu Antarkitiki* 13–42 (Izdatel'stvo Nauka, Moskva, 1971).
- ¹¹ Edwards, C. W. *Br. Antarct. Surv. Bull.* (in the press).
- ¹² Griukurov, G. E., Krylov, A. Ya. & Silin, Yu. I. *Dokl. Akad. Nauk. S.S.S.R., Geology* 172, 168–71 (1967).
- ¹³ Lowrie, W. & Hayes, D. E. *Initial Rep. DSDP* 28, 869–78 (1975).
- ¹⁴ Bailey, I. W. & Sinnott, E. W. *Science* 41, 831–834 (1915).
- ¹⁵ Drewry, D. J. *J. geol. Soc., Lond.* 131, 255–273 (1975).
- ¹⁶ Kemp, E. M. & Barrett, P. J. *Nature* 258, 507–508 (1975).

Megafaunal biomass in the deep sea

THE conventional notion of the deep-sea ecosystem has been one where a rain of fine organic particles from above provides food for a great diversity of small creatures living in and on the sediments^{1–3}. These, the meiofauna and macrofauna, live as deposit feeders, suspension feeders, and, in lesser proportion, as carnivores preying on other members of the fauna. The macrofauna in turn are thought to provide food for the generally more carnivorous megafauna, the echinoderms, decapods, and fishes. Although no data have been published concerning the abundance of the deep-sea megafauna in terms of biomass, the assumed trophic relationship and the Eltonian concept has led to the common feeling that the biomass of this component must be small in comparison to the meiofaunal and macrofaunal biomass⁴. Our data show that, on the contrary, the megafaunal biomass approaches that measured in the macrofauna. This observation re-focuses attention on the question of food supply to the deep sea, and implies that previously

suggested falls of large dead animals from the pelagial^{5,6} may be important.

As part of a general study of the distribution and ecology of deep benthic communities, we have made 116 trawl collections on the continental slope, rise, and abyss in the western Atlantic south of New England. Our principal gear for deep bottom trawling has been a 41-ft Gulf of Mexico shrimp trawl of 2.5-cm stretch mesh and fished with steel V doors. We have used a smaller 16-ft semiballoon trawl at slope depths. The animals obtained have been identified to species, counted, and weighed wet, usually after preservation in 10% formalin. From these data, we have determined mean wet weight per specimen for every taxon as a function of depth. Absolute abundance estimates were derived from photographic series made with a pair of E.G. & G. cameras mounted on DSRV ALVIN and fired at a rate of one frame per 10 s as the submersible cruised along a measured transect. Over 3,500 frames have been analysed from eight dives (436, 583, 586, 587, 589, 590, 591 and 592) in the same area where the trawling was carried out. Absolute abundance times mean weight within the appropriate depth interval gives the biomass. The data for deep benthic fishes, which comprise as a group about 40% of the total wet weight of megafaunal biomass in our trawl collections, are shown in Table 1.

The only previously available data concerning macrofaunal biomass off southern New England does not differ significantly ($P>0.65$) from the megafaunal (fish) data of Table 1. Most of these data, however, were taken with anchor dredges and seem to underestimate biomass considerably. New data on macrofaunal biomass off southern New England, obtained from 57 DSRV-operated box-core samples sieved through a 0.42-mm screen, were therefore used, and are plotted in Fig. 1. Total megafaunal biomass is obtained by extrapolation from the data of Table 1, using the actual weight percentage comprised by fish in each of the 46 trawls as the conversion factor.

Figure 1 compares macrofaunal and megafaunal biomass in the deep water south of New England. They are essentially of the same order of magnitude. The assumption that the biomass of the larger animals is relatively insignificant⁴ seems to be unfounded. Unless turnover rates in the macrofauna are relatively very high—and all the evidence suggests that this is not the case^{8,9}—the megafaunal biomass cannot be supported by the macrofauna. The megafauna must therefore depend to a considerable extent on food arriving from pelagic regions in the form of large, fast-falling packets, that is, the bodies of fishes, whales, squids and decapods. Based on observations made with a baited deep-sea camera, it has been suggested that this source could form an important fraction of the nutrition for the deep-sea

Table 1 Abundance and biomass of demersal fishes on the continental slope and rise

Mean depth (m)	Area surveyed (m ²)	No. per 1,000 m ²	No. of trawls	No. of specimens	Total weight (g)	Mean weight	Density (g m ⁻²)
497	1,548	56.7*	8	451	21,599	47.9	2.72
996	2,580	27.1*	4	24	2,173	90.5	2.45
1,329	1,517	4.6*	6	158	21,466	135.9	0.63
1,507	2,614	26.2*	4	94	20,757	220.8	5.78
1,768	6,320	12.8	2	315	61,370	194.8	2.49
1,800	2,101	4.2*	3	317	61,830	195.2	0.83
1,814	1,820	11.6*	2	18	3,041	168.9	1.96
1,815	3,372	8.9	1	2	500	250.0	2.23
1,960	12,524	7.9	2	52	26,946	518.2	4.09
2,066	1,656	13.3	2	85	29,797	350.6	4.66
2,332	7,666	3.8	3	118	61,308	519.6	1.97
2,426	24,712	6.1	2	27	8,795	325.7	1.99
2,474	15,948	4.4	3	60	25,676	427.9	1.88
2,786	2,786	4.2	4	127	35,382	278.6	1.17

Columns 1–3 contain data from the submersible; columns 4–7 are from trawls at the same approximate depths. Abundance data marked with an asterisk are from Grassle *et al.*¹⁶.

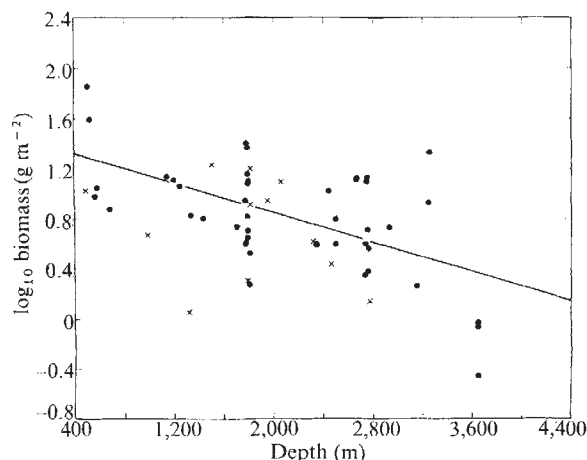


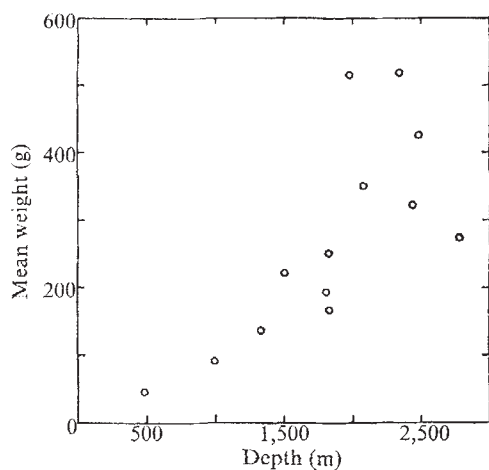
Fig. 1 Biomass against depth in macrofaunal infauna (O) and megafauna (X). The regression line is for the infaunal data ($\log_{10} B (g\ m^{-2}) = 1.44 - 0.41Z(km)$; $r = -0.58$, $P < 0.001$).

benthic ecosystem³. Our data support this idea. Further support is found in studies of the food habits of *Coryphaenoides armatus*, the dominant macrourid fish on the continental rise. This species accounts for as much as 80% of the fish biomass there and depends on large pelagic creatures for its food^{10,11}.

The need to search wide barren areas for a few randomly occurring food items and the fact that the metabolic demands of a large animal are less per unit weight than for a small one¹² should select for large efficient seekers in the deep-sea megafauna. Figure 2 shows the relationship between mean individual fish weight and depth, based on the trawled material of Table 1. There is a dramatic increase at the beginning of the rise near 2,000 m. The 'bigger-deeper' phenomenon, shown here for all fish taxa combined, applies as well within individual species and seems to be quite common in deeper-living marine fishes¹³. (Our data indicate that only a few megafaunal invertebrates are 'bigger-deeper', and the red crab *Geryon* is 'smaller-deeper' (ref. 14.) The efficiency of large deep-benthic fishes is evidenced by the rapidity with which they assemble to baited cameras³.

Based primarily on studies of the meiofaunal and macrofaunal elements of the deep-sea benthos, it has been suggested that small forms should dominate food-limited associations¹⁵. Our consideration of the megafaunal fishes implies just the opposite. The point is that both life styles are possible in the deep sea, particularly in the case where

Fig. 2 Mean weight of fish against depth. Data from Table 1. The regression is significant ($W(g) = 0.18Z(m) - 57.3$; $r = 0.73$, $P < 0.002$).



the faunal components are relatively independent of one another. Where mobility is important, large size will be selected for, but where the ability to live in a fine-grained sediment and to feed on a thin organic layer is important, small size will be the rule.

We thank R. Carney, J. F. Grassle, R. R. Hessler, H. L. Sanders, and R. D. Turner for their comments on the manuscript. C. H. Clifford and P. T. Polloni assisted in the photo analyses and at sea. D. M. Cohen was the principal observer on all ALVIN dives, and has provided help and encouragement of this work in all its stages. Support came from the NSF and the National Oceanic and Atmospheric Agency.

RICHARD L. HAEDRICH
GILBERT T. ROWE

Woods Hole Oceanographic Institution,
Woods Hole, Massachusetts 02543

Received 30 May; accepted 13 July 1977.

- Marshall, N. B. *Aspects of Deep-Sea Biology* (Hutchinson, London, 1954).
- Menzies, R. J. *Int. Rev. ges. Hydrobiol.* 47, 339-345 (1962).
- Hessler, R. R. in *The Biology of the Oceanic Pacific* (ed. Miller, C. B.) (Oregon State University Press, Corvallis, 1974).
- Grassle, J. F. & Sanders, H. L. *Deep-Sea Res.* 20, 643-659 (1973).
- Dayton, P. K. & Hessler, R. R. *Deep-Sea Res.* 19, 199-208 (1972).
- Isaacs, J. D. & Schwartzlose, R. A. *Sci. Am.* 233, 85-91 (1975).
- Rowe, G. T., Polloni, P. T. & Horner, S. G. *Deep-Sea Res.* 21, 641-650 (1974).
- Smith, K. L. & Teal, J. *Science* 179, 282-283 (1973).
- Wirsén, C. O. & Jannasch, H. W. *Environ. Sci. Technol.* 10, 880-886 (1976).
- Haedrich, R. L. & Henderson, N. R. *Deep-Sea Res.* 21, 739-744 (1974).
- Pearcy, W. G. & Ambler, J. W. *Deep-Sea Res.* 21, 745-759 (1974).
- Hemmingsen, A. M. *Rep. Sleno Mem. Hosp. Nordisk Insulin Lab.* 9, 1-110 (1960).
- Bullis, H. & Struhsaker, P. Q. *J. Florida Acad. Sci.* 33, 43-76 (1970).
- Wigley, R. L., Theroux, R. B. & Murray, H. E. *Mar. Fish. Rev.* 37, 1- (1975).
- Thiel, H. *Int. Rev. ges. Hydrobiol.* 60, 575-606 (1975).
- Grassle, J. F., Sanders, H. L., Hessler, R. R., Rowe, G. T. & McClellan, T. *Deep-Sea Res.* 22, 457-481 (1975).

Ultradian rhythms in urine flow in waking humans

THERE is evidence that neural oscillators may regulate the flow of urine, Mandell *et al.*¹ demonstrated ultradian rhythms in urine flow in catheterised patients during sleep, synchronised with the sleep REM-nonREM cycles (REM, rapid eye movement). They attributed these rhythms to rhythmic secretion of antidiuretic hormone (ADH) during sleep. Others have demonstrated episodic secretion of ADH during sleep but no correlation with REM-nonREM cycles². Reports³⁻¹⁰ of ultradian rhythms of 90-100 min analogous to the REM-nonREM cycles in waking subjects in physiological, endocrine and psychological processes, suggested the existence of waking ultradian rhythms in the flow of urine. We report here two experiments which demonstrate these rhythms.

In experiment 1 eight healthy, male college students aged 18-20 yr, paid to participate, and unaware of the hypothesis, avoided alcohol and drugs for 24 h preceding the study. To avoid catheterisation, subjects were required to drink a constant amount of fluid and then to urinate as much as possible every 10 min for the next 10 h. Each began between 10:00 h and 13:00 h. Two subjects drank 30 ml of commercial calorie balanced liquid food, two drank 50 ml of liquid food, and four drank 30 ml of liquid food plus 30 ml of water. Each sample was collected into a graduated cylinder and volume was measured to the nearest ml. After each voiding subjects were given a brief perceptual task lasting approximately 40 s (ref. 10). When not voiding subjects were required to remain seated but could read, write, listen to music or talk to each other (all were tested in pairs).

In the second experiment, 12 healthy, male college students, aged 19-25 yr were tested in a similar way. Subjects drank 25 ml of fruit juice plus 25 ml of water. Part of each 10-min urine sample was frozen immediately for analysis. As before, after each voiding, subjects were

given a brief perceptual task and then remained seated until the next trial.

In spite of the heavy experimental demands 17 out of the 20 subjects provided urine at all 60 attempts. Data for three subjects, two from the first experiment and one from the second, had to be discarded because they had failed to urinate on 10 consecutive attempts. Prominent ultradian rhythms were visible in the flow of urine for most subjects during the 10 h. Figure 1 shows the record of a representative subject in the second experiment. The mean coefficients of variation (s.d./x) for urine volumes were 34.5% and 42% for the first and second study respectively; the overall range varied between 72% and 24%. Na^+ and K^+ concentrations measured by flame photometry for 11 subjects of the second experiment, and urinary osmolality measured with a freezing point osmometer for seven subjects, showed identical rhythms reciprocal in-phase to the rhythms in urine flow.

The statistical significance of the ultradian rhythmicity was determined by time series analysis and inferential statistical procedures. First, the mean for each time series of urine volumes, K^+ and Na^+ concentrations and urine osmolality were subtracted from each data point, and the linear trend was eliminated. Then, autocorrelation functions were computed and the variance spectra were estimated^{11,12}. Spectra were calculated for 10 orthogonal frequencies from 0 to 64.8 cycles per day, in increments of 7.2 cycles per day. For each variable, spectra were hanned (hanning is a smoothing procedure to increase the reliability of the spectral estimates) individually, normalised and then averaged across all subjects. Cross correlation functions, hanned, normalised cross spectra and cross spectral phase angles were also computed for the same 10 spectral frequencies between all pairs of variables for each subject. Average spectra peaked at 14.4 cycles per day (corresponding to 1 cycle every 80–133 min) for all variables (Fig. 2). We had predicted that variance would peak in the spectra at 14.4 cycles per day, so we compared the variance at this frequency with variance at the adjacent spectral frequencies and with the mean variance at the rest of the spectral frequencies, by one-tailed *t* tests. All six comparisons yielded significant *t* ratios in the expected direction ($P < 0.05$, or better).

The ultradian rhythms in urine flow were clearly out of phase with electrolyte concentrations and osmolality rhythms. Pearson product moment correlations between volume time series and electrolyte concentrations and osmolality were negative for all subjects, and differed significantly from zero ($P < 0.001$) for all three variables. Similarly, cross-spectral phase angle analysis revealed that K^+ , Na^+ and osmolality were in-phase with each other and about 180° out-of-phase with urine volume.

Our results demonstrate prominent ultradian rhythms in urine flow of waking subjects. Although we do not

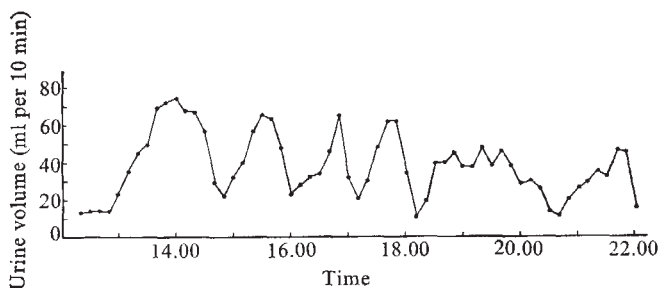


Fig. 1 Time series constructed from raw data of urine volumes sampled every 10 min from 1200 until 2200. Spectral analysis revealed a dominant ultradian periodicity of approximately 80–133 min per cycle.

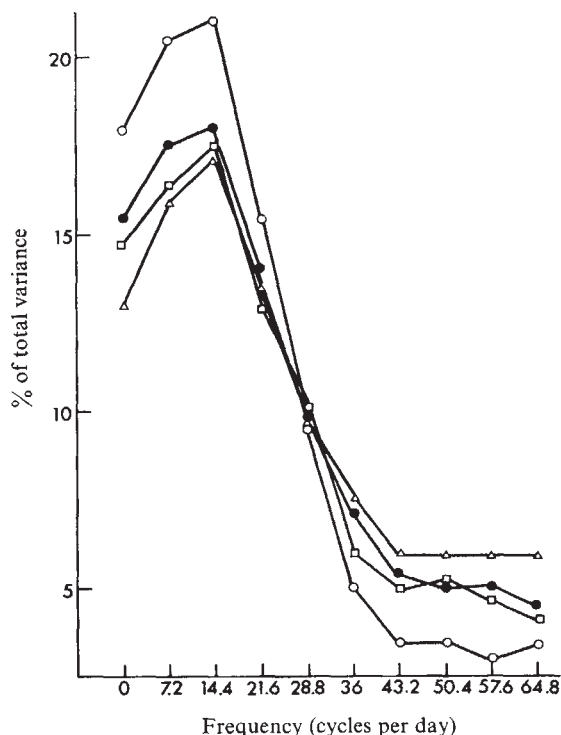


Fig. 2 Average spectra for urine volumes ($\square$, $n = 17$), osmolality ($\circ$, $n = 7$), K^+ ($\bullet$, $n = 11$) and Na^+ ($\triangle$, $n = 11$) concentrations. All spectra peaked at 14.4 cycles per day (corresponding to 80–133 min per cycle).

know whether these were generated by the oscillatory processes underlying the REM–nonREM cycles and the sleep urinary rhythms, the reciprocal phase relationships between volumes and osmolality indicates that rhythmic secretion of ADH is responsible for the rhythms in urine flow. The reports that all anterior pituitary hormones are secreted episodically during sleep and wakefulness support such an explanation¹³. But to test this endocrinological explanation we are repeating this study, making ADH measurements concomitant with urine sampling.

The accumulation of evidence of ultradian rhythms in multiple organismic processes during both sleep and wakefulness suggest that ultradian rhythms reflect an endogenous 'biological hour' of as yet unknown function and origin, similar to the well documented 20–28-h circadian 'biological day'.

This work was supported by a grant to P.L. from the Israel Center for Psychobiology. We thank Ms N. Adress and F. Krasovec for technical assistance.

P. LAVIE*

Department of Behavioral Biology,
Aba Khoushy School of Medicine,
Gutwirth Building,
Technion City, Haifa, Israel

D. F. KRIPKE

Department of Psychiatry,
University of California,
San Diego, California

Received 7 February; accepted 28 June 1977.

*To whom reprint requests should be addressed.

- Mandell, A. J. *et al. Science* **151**, 1558–1560 (1966).
- Rubin, R. T. *et al. Endocr. Res. Commun.* **2**, 459 (1975).
- Friedman, S. & Fisher, C. J. *Am. Psychoanal. Ass.* **15**, 317–343 (1967).
- Kripke, D. F. *Psychosom. Med.* **34**, 221–234 (1972).
- Oswald, J., Merrington, J. & Lewis, H. *Nature* **225**, 959–960 (1970).
- Lavie, P., Lord, J. W. & Frank, R. A. *Behav. Biol.* **11**, 373–379 (1974).
- Lavie, P., Levy, C. M. & Coolidge, L. F. *Physiol. Psychol.* **3**, 144–146 (1975).
- Lavie, P. *Chronobiologia* **3**, 214–218 (1976).
- Hiatt, J. F. & Kripke, D. F. *Psychosom. Med.* **37**, 320–325 (1975).

- ¹⁰ Lavie, P., Lord, J. W. & Frank, R. A. *Behav. Biol.* **11**, 373-379 (1974).
¹¹ Halberg, F. & Panofsky, H. *Exp. Med. Surg.* **19**, 284-309 323-328 (1961).
¹² Blackman, B. R. & Tukey, J. W. *The Measurement of Power Spectra* (Dover, New York, 1958).
¹³ Weitzman, E. D., Boyar, R. M., Kapen, S. & Hellman, L. in *Rec. Prog. Horm. Res.* **31**, 399-445 (1975).

Natural selection at the α -GDH locus in *Drosophila*

THE molecular basis for the maintenance of a gene-enzyme polymorphism in natural populations of *Drosophila melanogaster* has been reported¹. The locus under analysis codes for the subunit of α -glycerophosphate dehydrogenase, (α -GDH; E.C. 1.1.1.8) a homodimeric enzyme: the primary function of this enzyme in adult muscle is to provide metabolic energy during flight²⁻⁷, and these alleles showed both spatial and temporal variations in frequency. A latitudinal cline was identified, in which the frequency of the α -GDH^S allele decreases southwards along the eastern US coast⁸. The α -GDH^F allele was also found to increase in frequency during cooler months in several New England populations⁶. We evaluated several biochemical parameters using partially purified enzyme from the three common genotypes and uncovered temperature-dependent differences in K_m , specific activity, and reaction rate constancy¹. Based on the population and biochemical data an argument was provided that environmental temperature was operating as a selective agent in demanding the relative frequencies of the two alleles at this metabolically important locus. We present here additional evidence to support this proposal, based on an evolutionary pattern of coincidence.

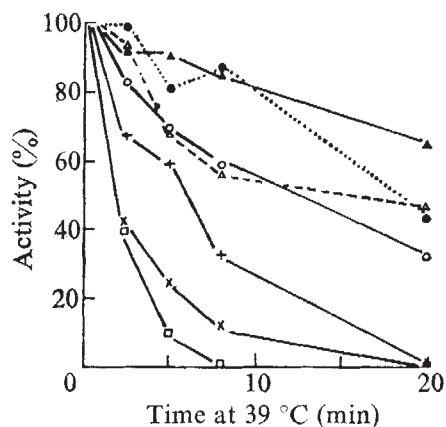


Fig. 1 Thermal stability of α -GDH. Partially purified enzyme (see ref. 1 for procedure) was isolated from 1-week-old *Drosophila* adults raised at 25 °C. Enzyme solutions at 39 °C were chilled, and assayed in a Guilford recording spectrophotometer, at 340 nm, for the reduction of NADH. $\circ$, *D. melanogaster* α -GDH^F; $\blacktriangle$, *D. melanogaster* α -GDH^S; $\square$, *D. virilis*; $\times$, *D. americana*; $+$, *D. arizonensis*; $\bullet$, *D. willistoni*; $\triangle$, *D. equinoxialis*.

The distinctive temporal and spatial patterns of allele frequency distribution, and the biochemical profiles discussed above, led us to refer to α -GDH^S as the cool-climate adapted allele, and α -GDH^F as the warm-climate adapted allele. This is somewhat misleading since α -GDH^F is always the most abundant allele, but as a relative statement the nomenclature is useful. We predicted that if α -GDH function is sensitive to environmental temperature a parallel response should be observed in temperature-dependent kinetic properties if we compared α -GDH of species inhabiting primarily temperate rather than tropical habitats. The observation of such parallel biochemical differentiation, in an evolutionary time frame, should carry considerable weight for the selection model in spite of our inability to experimentally demonstrate the exact *in vivo* significance of differences found in cuvette assays. Since every other *Drosophila* species reported on the essentially monomorphic at the α -GDH locus⁹⁻¹² our analysis would not be confounded by interspecific variation in our additional species samples.

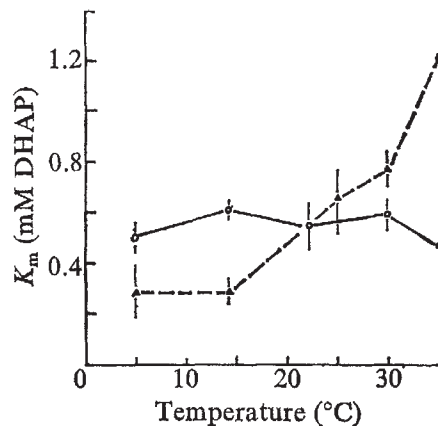


Fig. 2 Apparent K_m values for the substrate, dihydroxyacetone phosphate (DHAP), for *D. melanogaster*, as a function of temperature. The strains S mark and ($\circ$) carrying α -GDH^F, and Swedish-C ($\triangle$) carrying α -GDH^S were employed. K_m at each temperature was determined from duplicate measurements of activity at each of 5 substrate concentrations by fitting $1/v$, $1/s$ data into a linear regression computer program. Concentrations of NADH were 120 μ M throughout. Assay temperatures were maintained in the cuvette chamber by a Haake circulating water bath. Bars around representative points indicate standard error values.

We examined three tropical species (*D. willistoni*, *D. arizonensis*, *D. equinoxialis*) and two temperate species (*D. virilis* and *D. americana*) (stocks provided by the Stock Centre, University of Texas, Austin: see ref. 13). Partially purified enzyme was prepared¹ from adult homogenates of each species as well as from strains of *D. melanogaster* homozygous for the two common alleles, and studied in several ways. We found that the electrophoretic mobility of α -GDH from each of the five additional species was indistinguishable from the α -GDH^S allele of *D. melanogaster* in starch gels, in a variety of electrophoretic conditions (pH 6.8 or 8.8, in 9% or 14% starch, in a Tris-citrate buffer system; pH 7.8 or 9.8, in 9% or 14% starch, in a Tris-borate buffer system)¹⁴. This pattern of conservation, with respect to electrophoretic mobility, for α -GDH has been noted before¹⁵. But, we could detect rather large differences among the species¹ enzymes by using thermal denaturation as a probe (Fig. 1). This observation supports a growing number of studies indicating that thermal stability analysis can detect hidden variation within a single electrophoretic mobility class¹⁶⁻¹⁹. We suspect that although

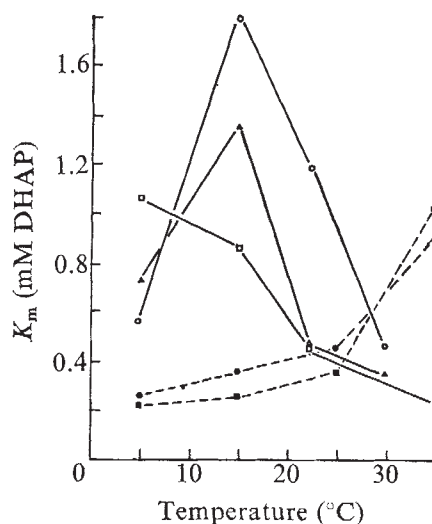


Fig. 3 Apparent K_m for DHAP for α -GDH obtained from: $\square$, *D. willistoni*; $\triangle$, *D. equinoxialis*; $\circ$, *D. arizonensis*; $\blacksquare$, *D. virilis* and $\bullet$, *D. americana*. Assay conditions as described in Fig. 2 legend.

closely related species (for example *D. virilis* and *D. americana*, or *D. willistoni* and *D. equinoxialis*) have similar thermostability properties it is unlikely that their primary structures are the same. It is interesting to note that, as a general rule, enzyme from the tropical *Drosophila* species is less heat sensitive than enzyme from the temperate species.

Kinetic studies (Fig. 2) confirmed the distinct K_m against temperature patterns found for the *D. melanogaster* 'fast' and 'slow' homozygotes¹. The α -GDH^F pattern showed temperature independence of affinity for substrate, while the α -GDH^S pattern displayed a temperature dependent pattern in which increased substrate affinity and temperature were inversely related. This latter type of pattern has been referred to as positive thermal modulation¹⁹, a mechanism by which, it is thought, reaction rate constancy is maintained over a range of environmental temperatures¹⁹. Figure 3 summarises the data obtained for the five new species. *D. virilis* and *D. americana*, both temperate species, showed K_m against temperature profiles remarkably like the α -GDH^S pattern. In contrast, the three tropical species had profiles which, while similar within the group, clearly differed from the temperate species¹ (α -GDH^F like) pattern. In contrast to α -GDH^F, in which K_m remained essentially constant, the tropical species had a pattern of negative thermal modulation (K_m decreases with increasing temperature) over most of the temperate range. In any event the tropical species' profile clearly more resembled the α -GDH^F pattern, especially at higher assay temperatures. Activation energies were calculated from the data, and all species and genotypes had quite similar values (7.7 ± 0.8). (Energies of activation determined by Arrhenius plots according to the formula in ref. 20).

The coincidence of K_m against temperature profiles described in the data, as predicted, seem based on species distribution rather than phylogeny. This observation strongly suggests that natural selection is operating at the α -GDH locus within the genus *Drosophila*, with temperature being the mediating agent. Note that this same conclusion has been drawn for α -GDH polymorphisms in two other insect species, *Colias*, a butterfly²¹, and *Cochliomyia*, the screw worm²². We feel that a general pattern may emerge among all flying insects with a carbohydrate based energy metabolism, in which environmental temperature will be shown critical in the adaptation and evolution of flight enzyme kinetics.

This work was supported by US NIH grant GM22866.

STAMATIS ALAHOTIS*

STEVE MILLER†

EDWARD BERGER‡

Biology Department, Dartmouth College
Hanover, New Hampshire 03755

Received 20 May; accepted 27 June 1977.

*Present address: Genetics Department, University of Patras, Greece.

†Present address: Genetics Department, North Carolina State University, Raleigh, North Carolina.

‡To whom correspondence should be addressed.

- ¹ Miller, S., Percy, R. W. & Berger, E. *Biochem. Genet.* **13**, 175–188 (1975).
- ² Sacktor, B. & Dick, A. J. *biol. Chem.* **237**, 3259–3262 (1962).
- ³ Sacktor, B. *Adv. Insect Physiol.* **7**, 267–347 (1970).
- ⁴ O'Brien, S. J. & MacIntyre, R. J. *Genetics* **71**, 127–138 (1972).
- ⁵ Grell, E. *Science* **158**, 1319–1320 (1967).
- ⁶ Berger, E. *Genetics* **67**, 121–136 (1971).
- ⁷ O'Brien, S. J. & MacIntyre, R. J. *Am. Naturalist* **102**, 193–205 (1968).
- ⁸ Johnson, F. & Schaffer, H. *Biochem. Genet.* **10**, 149–163 (1973).
- ⁹ Kojima, K., Gillespie, J. & Tobari, Y. N. *Biochem. Genet.* **4**, 627–637 (1970).
- ¹⁰ Zouros, E. *Nature* **254**, 446–448 (1975).
- ¹¹ Barker, J. S. F. & Mulley, J. C. *Evolution* **30**, 213–233 (1976).
- ¹² Powell, J. in *Evolutionary Biology* **8**, 79–119 (eds Dobzhansky, T., Hockett, M. K. & Steere, W. C.) (Plenum, New York, 1976).
- ¹³ Patterson, J. T. & Stone, W. *Evolution in the Genus Drosophila*. (Macmillan, New York, 1952).
- ¹⁴ Shaw, C. R. & Prasad, R. *Biochem. Genet.* **4**, 297–320 (1970).
- ¹⁵ Hubby, J. L. & Throckmorton, L. H. *Am. Nat.* **102**, 193–205 (1968).
- ¹⁶ Bernstein, S., Throckmorton, L. H. & Hubby, J. L. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3928–3931 (1973).
- ¹⁷ Singh, R. S., Lewontin, R. C. & Felton, A. A. *Genetics* **84**, 609–629 (1976).
- ¹⁸ Singh, R. S., Hubby, J. L. & Lewontin, R. C. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1808–1810 (1974).
- ¹⁹ Hockachka, P. W. & Somero, G. *Strategies of Biochemical Adaptation* (Saunders, Philadelphia, 1973).
- ²⁰ Robert, M. & Gray, I. *Comp. Biochem. Physiol.* **42B**, 389–402 (1972).
- ²¹ Johnson, G. B. *Stadler Genet. Symp.* **7**, 91–111 (1975).
- ²² Bush, G. L., Neck, R. W. & Kitto, G. B. *Science* **193**, 491–493 (1976).

Genetic control of cell division in yeast cultured at different growth rates

HARTWELL *et al.*^{1,2} have isolated a number of temperature-sensitive mutants blocked at various stages of the cell division cycle of *Saccharomyces cerevisiae*. A characteristic feature of any temperature-sensitive cell cycle mutant is its execution point in the cell cycle, that is, the point in the cycle after which cells when shifted to the restrictive temperature undergo one further cell division. If cells are shifted to the restrictive temperature before the execution point, they are unable at the elevated temperature to complete any cell division. The execution point for a particular mutant is taken to be the stage in the cycle that the defective gene completes its function. The execution point of mutant strain *cdc28* is at an earlier stage in the cycle than other cell cycle mutants³. Detailed investigations of a number of cell cycle mutants have suggested that the events in early G₁ that culminate in the execution of the *cdc28*-mediated step represent 'start'. There is evidence that passing 'start' in the cycle represents a point of commitment to division. Before the execution of the *cdc28*-mediated step, 'start', yeast is capable of several developmental programmes but after this step it is committed to the mitotic cell cycle⁴. We report here that at generation times varying from 2.1–6.18 h 'start' occurs approximately 2 h before division. At slower growth rates the length of time from 'start' to division increases somewhat. These results are similar to the finding in the bacterium *Escherichia coli* that the time from the initiation of DNA synthesis to cell division is independent of growth rate except at slow growth rates⁵.

A number of authors^{6–7} have shown that if one knows the percentage of cells in an asynchronous culture that are past a particular event in the cell cycle, the stage in the cycle that the event occurs can be determined. The execution point of a temperature-sensitive cell cycle mutant can be calculated from the percentage increase in cell number after such a mutant is shifted from the permissive to the restrictive temperature, since this reflects the percentage of cells that are past the execution point at the time of the shift. An allowance has to be made for the fact that the age distribution of cells throughout the cycle is skewed⁸, using an equation which describes the age distribution⁷.

A strain that carries the mutation *cdc28* has been cultured at different growth rates at 24 °C in batch cultures by altering media composition and in chemostat cultures by altering the flow rate of the limiting nutrient, glucose. Cultures at different growth rates were shifted to 38 °C and cell numbers after the shift were monitored. Fig. 1 shows the effect of shifting *cdc28* with a generation time of 6.18 h to 38 °C. Cell numbers continue to increase for a time until cell division stops and a plateau in cell numbers is reached. The percentage increase in cell numbers after the temperature shift is equivalent to the percentage of cells past the *cdc28* execution point and from this data the stage in the cycle of the execution point is determined using the equation of Howell and Nabiloff⁷. In the experiment described in Fig. 1 the execution point for the *cdc28* gene product occurs at 0.42 of the cell cycle.

Table 1 shows the percentage increase in cell numbers after *cdc28* cultured at different growth rates is shifted to 38 °C. The cell number increase is greatest at the fastest growth rates and the percentage increase is least at the slowest growth rates. Clearly, if in an exponential culture many cells at the permissive temperature are past the *cdc28* execution point, this event occurs at an early stage of the cycle. Conversely, if only a few cells

Table 1 Effect of specific growth rate on the stage and time in the cell cycle of the *cdc28* gene product execution point

Specific growth rate (h^{-1})	Generation time (h)	Percentage increase in cell numbers after shift to 38 °C	Stage in the cell cycle of the <i>cdc28</i> execution point	Time before cell division of the <i>cdc28</i> execution point (h)
0.330	2.1	133	-0.12	2.5
0.254	2.73	85.7	0.11	2.4
0.180	3.85	57	0.35	2.5
0.112	6.18	50	0.42	3.58
0.032	21.6	21.4	0.72	6.05

Haploid *Saccharomyces cerevisiae* cells of strain H.185.3.5 carrying the *cdc28* mutation were grown at different steady state specific growth rates in a glucose limited chemostat at 24 °C in the media described in Fig. 1. Samples at different specific growth rates were removed from the chemostat, and diluted in fresh medium at 38 °C. Cell number was monitored at intervals as described in Fig. 1, until cell division at the restrictive temperature ceased. The percentage of cells that divided at the restrictive temperature was used to calculate the stage and time in the cycle of the *cdc28* gene product execution point using the equation described in Fig. 1.

are past this event at the time of the shift then it occurs at a late stage of the cycle. Table 1 shows that stage in the cycle of *cdc28* execution point is dependent on growth rate such that as the growth rate is slowed the execution point occurs later and later in the cycle. The time from the *cdc28* execution point to cell division is constant in cells growing with generation times of 2.1, 2.73 and

execution point for *cdc28* occurs in the previous cycle to the division which it controls, is in keeping with the view that the *cdc28* gene product decays slowly at the restrictive temperature. Others have suggested that the *cdc28* mutation is 'leaky'.

Haploid yeast cells of 'a' mating type produce a small polypeptide ' α factor' of molecular weight 1,400, which

Table 2 Effect of specific growth rate on the stage and time in the cell cycle of the α factor-sensitive step

Specific growth rate (h^{-1})	Generation time (h)	Percentage increase in cell numbers in the presence of α factor	Stage in the cell cycle of the α factor factor-sensitive step	Time before cell division of the α factor-sensitive step
0.256	2.7	56.9	0.13	2.35
0.20	3.46	60	0.33	2.32
0.15	4.62	42.8	0.49	2.36
0.112	6.18	28.5	0.63	2.29
0.076	9.1	26.6	0.65	3.18

Haploid *Saccharomyces cerevisiae* cells of strain H185.3.4 carrying the *cdc28* mutation were grown at different steady-state specific growth rates in a glucose limited chemostat at 24 °C in the media described in Fig. 1. Samples at different specific growth rates were removed from the chemostat, and diluted in fresh medium at 24 °C containing α factor. The α factor was purified as described previously⁸, and a concentration of α factor was chosen that produced greater than 95% unbudded cells and that inhibited budding for at least 8 h. Cell number was monitored at intervals as described in Fig. 1, until cell division in the presence of α factor ceased. The percentage of cells that divided in the presence of α factor was used to calculate the stage and time in the cycle of the α factor sensitive step using the equation described in Fig. 1.

3.85 h but at slower growth rates it expands in time. A possible explanation for this expansion is that the *cdc28* thermosensitive gene product decays quite slowly at 38 °C. If this is true then the gene product will continue to function for some time at the restrictive temperature, and consequently these cells will continue to divide. The result of this is that the execution point for *cdc28* will seem to be earlier in the cycle than the time at which the gene product normally completes its function. Our observation (Table 1) that at fast growth rates the

contains a number of common amino acids⁸. When the α factor is added to cells of 'a' mating type, the latter are arrested as small unbudded cells before the initiation of DNA synthesis. The point of α factor-mediated cell cycle arrest has been mapped within the sequence of three gene functions before the initiation of DNA synthesis, and it has been shown that the α factor-sensitive step is the same as that mediated by the *cdc28* gene product³.

Because of the uncertainty attached to precise location

Table 3 Effect of growing *Saccharomyces cerevisiae* in different media supporting different growth rates on the stage and time in the cell cycle of the α factor-sensitive step

Culture medium	Specific growth rate (h^{-1})	Generation time (h)	Percentage increase in cell numbers in the presence of α factor	Stage in the cell cycle of the α factor-sensitive step	Time before cell division of the α factor-sensitive step
YEPD	0.247	2.8	57.0	0.35	1.83
Proline	0.208	3.33	50.0	0.42	1.93
YEPG	0.134	5.16	30.5	0.62	1.96
Ethanol	0.084	8.3	22.0	0.71	2.41
Acetate	0.082	8.5	21.5	0.72	2.38

Haploid wild-type *Saccharomyces cerevisiae* strain A364a were grown on different media at 24 °C. The media were: YEPD, containing in 1 l of distilled water, 10 g yeast extract, 20 g bacto-peptone, 20 g glucose; proline, containing in 1 l of distilled water, 2 g proline, 20 g glucose, 1.4 g yeast nitrogen base without amino acids; YEPG, containing in 1 l of distilled water, 10 g yeast extract, 20 g bacto-peptone, 20 ml glycerol; ethanol, containing in 1 l of distilled water, 20 ml ethyl alcohol 95%, 5 g ammonium sulphate, 6.7 g yeast nitrogen base without amino acids; and acetate, containing in 1 l of distilled water, 20 g potassium acetate, 6.7 g yeast nitrogen base without amino acids. All these media, except YEPG and YEPD, also contained 0.04 g tyrosine, 0.04 g lysine, 0.04 g histidine, 0.01 g uracil and 0.1 g adenine per l, trace elements and vitamins. Cultures were grown on rotary shakers at 24 °C. These media support generation times of 2.8, 3.33, 5.16, 8.3 and 8.5 h respectively. Samples at different growth rates were diluted in fresh YEPD medium at 24 °C containing α factor. Cell number was monitored at intervals as described in Fig. 1, until cell division ceased in the presence of α factor. The percentage of cells that divided in the presence of α factor was used to calculate the stage and time in the cell division cycle of the α factor-sensitive step using the equation described in Fig. 1.

in time of the *cdc28* execution point, we have determined the stage and time in the cycle of the α factor-sensitive step for cells cultured at different growth rates in the chemostat and treated with α factor; the percentage of cells that divide in the presence of α factor was used as before to determine the stage in the cycle of the α factor-sensitive step. Table 2 shows that for cells cultured at different growth rates in the chemostat the α factor step was at the beginning of the cycle at fast growth rates, but as the growth rate was slowed it moved to later stages of the cycle. In terms of time, however, it occurred at generation times less than 6.18 h, a constant time before cell division. Similar results were obtained when the time of the α factor-sensitive step was investigated in cells grown at different growth rates using different media in batch culture (Table 3). At longer generation times the time from 'start' to cell division increases; for instance, at a generation time of 9.1 h the time from

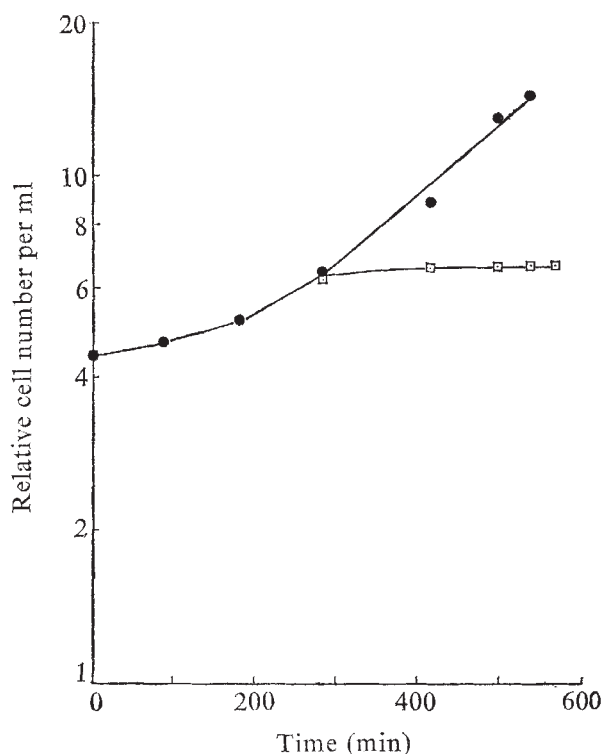


Fig. 1 Effect on cell division of shifting *cdc28* previously grown with a doubling time of 6.18 h at 24 °C in a chemostat to the restrictive temperature 38 °C. Haploid *Saccharomyces cerevisiae* cells of strain H185.3.4 carrying the *cdc28-1* mutation were grown at 24 °C in a chemostat (L.H. Engineering) at a steady-state specific growth rate of 0.112 h⁻¹ (generation time 6.18 h). The chemostat medium contained 10 g yeast extract, 10 g bacto-peptone, 10 g glucose as limiting nutrient, 5 g ammonium sulphate, 2 g ammonium phosphate, 1 g magnesium sulphate and 100 mg adenine in 1 litre of distilled water at pH 5.5. Samples were removed from the chemostat diluted in fresh medium and one sample was incubated at 24 °C (●) and the other at 38 °C (□) in a shaking water bath. Samples were removed from both cultures at intervals, diluted in Isoton (Coulter Electronics) and cell numbers determined in an electronic particle counter (Coulter Electronics) after 90 s treatment with an MSE homogeniser to break up clumps. The percentage increase in cell numbers after the shift to 38 °C (in this case 50%) was used to determine the stage in the cycle of the *cdc28* execution point using the equation

$$X = 1 - \frac{\ln(N/N_0)}{0.693}$$

where X is the stage in the cycle of the execution point, N_0 is equal to the cell number at the time of the temperature shift and N is equal to the final cell number at the restrictive temperature⁹. The stage in the cell cycle of the execution point was calculated to be 0.42.

'start' to cell division was 3.18 h (Table 2). The results presented in Table 1 can also be interpreted to indicate that at very slow growth rates the time from 'start' to cell division lengthens. If the decay time for the *cdc28* gene product is the same at varying growth rates, a constant amount should be subtracted from the time between the apparent execution point and cell division in the last column of Table 1. This would leave this time constant for generation times between 2.1 h and 3.85 h; and if, for example, the decay time took 0.2 h the time from the real execution point to cell division would be nearly the same as the equivalent 'start-division' time in the last column of Table 2. This assumption of a constant decay time, however, would not explain the increased 'start-division' time of for instance 6.05 h found for cells grown with a generation time of 21.6 h (Table 1).

These results indicate that for cells growing with generation times of less than 6.18 h the cell cycle can be divided into an expandable phase before the α factor-sensitive step, the length of which is dependent on growth rate, and a constant phase from the α factor-sensitive step to cell division, the length of which is independent of growth rate. As the α factor step is the same as that mediated by the *cdc28* gene product, which is thought to represent 'start', our results indicate that once cells pass 'start' they proceed to cell division in a constant time regardless of growth rate. Cells take a variable time to reach 'start' at different growth rates. It is interesting to note that when yeast populations were grown at long generation times in a chemostat the budded phase of the cycle was slightly longer than during rapid growth while the unbudded phase was selectively lengthened¹⁰.

At longer generation times the 'start-division' time increased although the pre-start period of the cycle was the main expandable phase. Our results are reminiscent of observations in *Escherichia coli* that the time from the initiation of DNA synthesis to cell division is constant at generation times faster than 60 min but at longer generation times there is a change in cell cycle timing controls.

We thank L. H. Hartwell for advice and yeast strains, the Berkeley Yeast Center for yeast strains, and J. Piggott for a gift of α factor. This work was supported by the Medical Research Council of Ireland and Arthur Guinness, Son and Co. Ltd.

M. N. JAGADISH
B. L. A. CARTER

Department of Genetics,
University of Dublin, Trinity College,
Dublin 2, Ireland

Received 24 May; accepted 19 July 1977.

- Hartwell, L. H. *Bact. Rev.* **38**, 164-198 (1974).
- Hartwell, L. H., Culotti, J., Pringle, J. R. & Reid, B. J. *Science* **183**, 46-51 (1974).
- Hereford, L. M. & Hartwell, L. H. *J. molec. Biol.* **84**, 445-461 (1974).
- Cooper, S. & Helmstetter, C. E. *J. molec. Biol.* **31**, 519-540 (1968).
- Hoffman, J. G. *Bull. Math. Biophys.* **11**, 139-144 (1949).
- Watanabe, I. & Okada, S. *J. Cell Biol.* **32**, 309-323 (1967).
- Howell, S. H. & Nabiloff, J. A. *J. Cell Biol.* **57**, 760-772 (1973).
- Cook, J. R. & James, T. W. in *Synchrony in Cell Division and Growth* (ed. Zeuthen, E.) 485-495 (Wiley-Interscience, New York, 1944).
- Duntze, W. D., Stotzler, E., Bucking-Throm, E. & Kalbitzer, S. *Eur. J. Biochem.* **35**, 357-365 (1973).
- Beck, C. & Meyenburg, H. K. von *J. Bact.* **96**, 479-486 (1968).

Blood flukes have a double outer membrane

THE blood fluke *Schistosoma mansoni* has an unusual tegumental outer membrane which consists of two lipid bilayers^{1,2}. We report here that the double outer membrane is a common feature of the Schistosomatidae (parasites of man which cause schistosomiasis) and also occurs in members of the two other families of blood flukes³, the

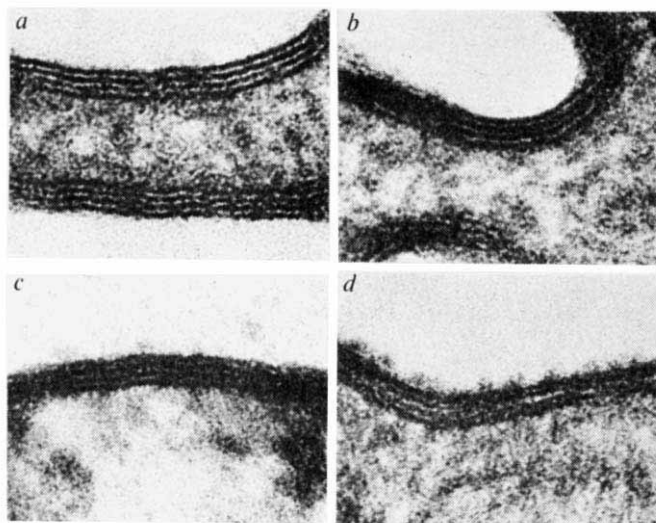


Fig. 1 Electron micrographs showing the double outer membrane of representative specimens from each of the three families of blood flukes. *a*, *Schistosoma japonicum* (Schistosomatidae); *b*, *Schistosoma margrebowiei* (Schistosomatidae); *c*, *Aporocotyle simplex* (Aporocotylidae); *d*, *Spirorchis* sp. (Spirorchidae). ($\times 240,000$).

Aporocotylidae (parasites of fish) and the Spirorchidae (parasites of turtles). In contrast, flukes (Digenea) belonging to families which inhabit the gut and associated body cavities of the host are limited by a single lipid bilayer. We suggest that the double outer membrane is an adaptation to the intravascular habitat.

Details of the flukes examined and their hosts and locations are presented in Table 1. Specimens were fixed for electron microscopy in glutaraldehyde, osmium tetroxide and uranyl acetate¹, dehydrated in ethanol and embedded in Araldite. Sections were stained with uranyl acetate and lead citrate.

In the Schistosomatidae the double outer membrane was present over virtually the entire surface of both male and female worms; a few small regions of the membrane

had a trilaminar or multilaminar appearance. In the fish and turtle blood flukes two lipid bilayers were found consistently over the whole surface of the parasite. The double outer membrane had the same appearance and identical dimensions in all the blood flukes examined (Table 1, Fig. 1). Each lipid bilayer was approximately 7 nm in thickness and the double outer membrane was 18 nm thick. Some specimens had a layer of diffuse material on the external surface of the double membrane, but we believe that this material originated from the host and did not represent a true glycocalyx. The flukes recovered from environments other than blood had only a single trilaminar outer membrane (single lipid bilayer) approximately 8 nm in thickness (Table 1, Fig. 2). Each of the non-blood flukes had a glycocalyx which varied in appearance from a thin but dense layer in *Fasciola hepatica* to a wide diffuse layer in *Macrolecithus papilliger*.

The advantage conferred by the presence of an additional membrane at the surface of the blood fluke may be related to the fact that any parasite living in the blood vascular system must be constantly exposed to the host's immune defence mechanisms in the form of leukocytes, complement and antibodies. The ability of adult schistosomes to evade the immune response may be due to the host-like antigenic determinants which are found on the worm's surface, and which may prevent the attachment of antibodies against the parasite^{4,5}. The double outer membrane of *S. mansoni* is formed immediately after host penetration^{1,6}, and before host antigens can be detected on the surface of the worm⁷. It has been suggested that the lipid molecules of the outer bilayer may be orientated for the insertion of host glycoproteins or glycolipids⁸. The idea that the two lipid bilayers may differ with respect to their lipid composition is supported by the fact that the outer bilayer is only visualised after uranyl acetate fixation^{1,8}. Rapid turnover and chemical unreactivity of the outer bilayer have also been suggested as possible mechanisms for countering antibody attack⁹. Turnover has been demonstrated *in vitro*⁹, but freeze-fracture studies have shown that most intercalated proteins occur in the outer lipid bilayer²—suggesting that the outer membrane may be more active than the inner membrane.

Table 1 Flukes examined by electron microscopy

Family	Genus and species	Host	Location
(1) Blood flukes			
Schistosomatidae	<i>Schistosoma haematobium</i>	Hamster	Hepatic portal vein
	<i>S. japonicum</i>	Hamster	Hepatic portal vein
	<i>S. matthei</i>	Hamster	Hepatic portal vein
	<i>S. bovis</i>	Hamster	Hepatic portal vein
	<i>S. leiperi</i>	Hamster	Hepatic portal vein
	<i>S. margrebowiei</i>	Hamster	Hepatic portal vein
	<i>S. intercalatum</i>	Hamster	Hepatic portal vein
	<i>S. haematobium</i> ♂	Hamster	Hepatic portal vein
	<i>S. intercalatum</i> ♀	Hamster	Hepatic portal vein
	} hybrid		Hepatic portal vein
Aporocotylidae	<i>Aporocotyle simplex</i>	Long rough dab	Gill arteries
	<i>A. spinosicanalis</i>	Hake	Heart
Spirorchidae	<i>Spirorchis</i> sp.	Painted turtle	Pulmonary venule
(2) Non-blood flukes			
Fellodistomatidae	<i>Steringophorus furciger</i>	Cod	Pylorus
Fasciolidae	<i>Fasciola hepatica</i>	Cow	Bile duct
Echinostomatidae	<i>Echinostoma caproni</i>	Mouse	Ileum
Notocotylidae	<i>Notocotylus tricephalis</i>	Chicken	Caecum
Dicrocoeliidae	<i>Dicrocoelium dendriticum</i>	Rabbit	Bile duct
	<i>Corrigia vitta</i>	Field mouse	Pancreatic duct
Acanthocolpidae	<i>Stephanostomum pristi</i>	Cod	Pylorus
Allocreadiidae	<i>Podocotyle atomon</i>	Scorpion fish	Rectum
	<i>Macrolecithus papilliger</i>	Minnow	Stomach
Lepocreadiidae	<i>Lepidopodon elongatum</i>	Cod	Pylorus
Zoogonidae	<i>Zoogonoides viviparus</i>	Dab	Intestine
Hemiuridae	<i>Hemiurus communis</i>	Flounder	Stomach
	<i>Derogenes varicus</i>	Cod	Stomach

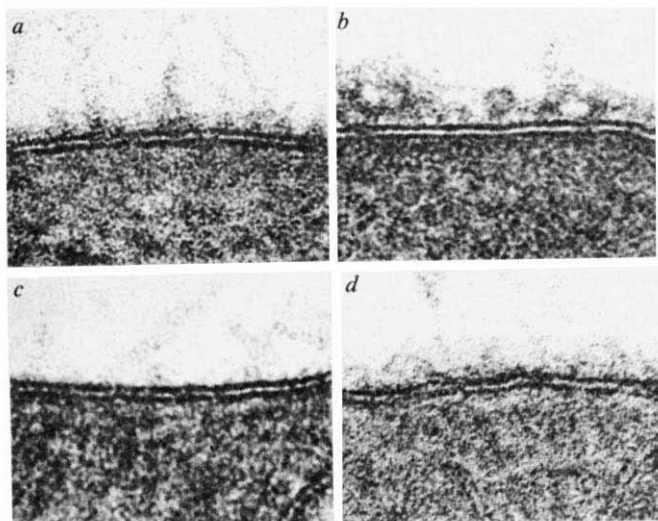


Fig. 2 Electron micrographs showing that the outer membrane of flukes recovered from environments other than the blood, consists of a single lipid bilayer. *a*, *Dicrocoelium dendriticum* (Dicrocoeliidae); *b*, *Derogenes varicus* (Hemiuridae); *c*, *Podocotyle atomon* (Allocreadiidae); *d*, *Echinostoma caproni* (Echinostomatidae). ($\times 240,000$).

We conclude therefore, that the double outer membrane is a characteristic feature of the blood flukes, that it consists of two conventional lipid bilayers with differing properties, and that it assists in protecting the parasite against the immunological response of the host.

We thank S. R. Smithers, V. R. Southgate, R. J. Knowles, C. James, J. Thulin, D. I. Gibson, M. Køie, M. Matricon, D. Düwel, J. W. Smith, A. R. Hockley, A. W. Pike, R. A. Matthews and M. R. L. Johnston for supplying specimens.

DIANE J. MCLAREN

Division of Parasitology,
National Institute for Medical Research,
Mill Hill, London NW7, UK

DAVID J. HOCKLEY

Division of Viral Products,
National Institute for Biological Standards
and Control,
Hampstead, London NW3, UK

Received 20 May; accepted 21 July 1977.

¹ Hockley, D. J. & McLaren, D. J. *Int. J. Parasit.* 3, 13–25 (1973).

² Hockley, D. J., McLaren, D. J., Ward, B. J. & Nermut, M. V. *Tissue Cell* 7, 485–496 (1975).

³ La Rue, G. R. *Expl. Parasit.* 6, 306–349 (1957).

⁴ Smithers, S. R., Terry, R. J. & Hockley, D. J. *Proc. R. Soc. B* 171, 483–494 (1969).

⁵ Clegg, J. A. *Ciba Fdn Symp.* 25, 161–183 (1974).

⁶ McLaren, D. J., & Hockley, D. J. *Parasitology* 73, 169–187 (1976).

⁷ McLaren, D. J., Clegg, J. A. & Smithers, S. R. *Parasitology* 70, 67–75 (1974).

⁸ Silva, M. T., Guerra, F. C. & Magalhães, M. M. *Experientia* 24, 1074 (1968).

⁹ Wilson, R. A. & Barnes, P. E. *Parasitology* 74, 61–71 (1977).

that macrophages whose phospholipid components were labelled with ^3H -arachidonic acid also synthesise and release ^3H -prostaglandins (PGs) in response to inflammatory stimuli. These observations are consistent with the findings that human macrophages on intrauterine devices⁸ and guinea pig macrophages responding to lymphokines⁹ release PGs.

It has been shown that tissues such as spleen¹⁰, heart and kidney¹¹, tumour cells¹² and platelets¹³ incorporate radiolabelled arachidonic acid into phospholipids and release labelled PGs. It is known that macrophage phospholipids contain a high proportion of this fatty acid¹⁴. We have found that unstimulated mouse peritoneal macrophages (Fig. 1) exposed to $1\ \mu\text{Ci}$ ^3H -arachidonic acid incorporate approximately 50% of the label into cellular phospholipids within 4 h. Extraction of either stimulated or unstimulated labelled cells showed that the majority of the isotope was incorporated into lecithin with smaller amounts found in phosphatidylethanolamine and triglycerides.

When cultures of unstimulated macrophages are maintained in a serum-free environment for 24 h, less than 3% of the label is released into the culture medium; only a small proportion of this has mobilities identical with standards of $\text{PGF}_{2\alpha}$, PGE_2 ,

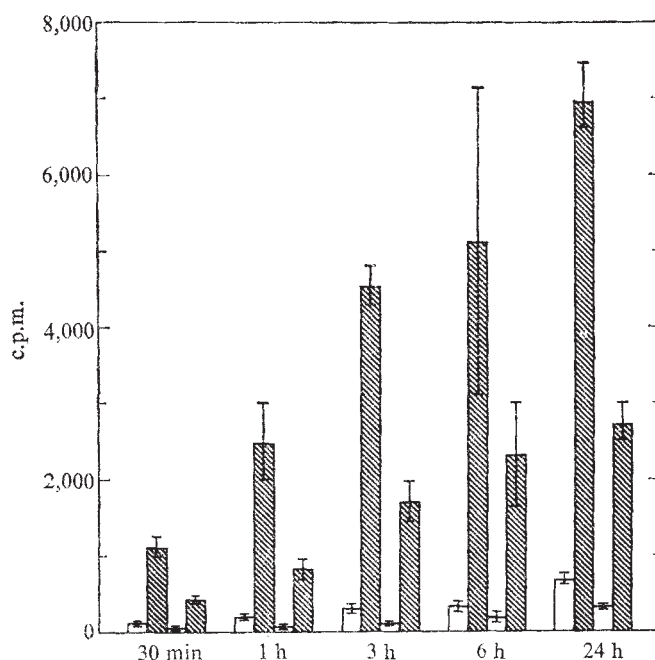


Fig. 1 Zymosan induces a time-dependent synthesis and release of ^3H -PGs from unstimulated macrophages. Macrophages were collected from outbred male SW-ICR mice by intra-peritoneal lavage with M199 media containing 1% heat-inactivated porcine serum (HIPS) and heparin $20\ \text{U ml}^{-1}$. The cells were plated at 4×10^6 cells per 60-mm dish and incubated for 2 h at 37°C in 5% CO_2 in air. The non-adherent cells were removed by washing and the adherent cells maintained overnight in M199 plus 10% HIPS. Cellular phospholipids were then labelled by incubating the cells in M199 plus 1% HIPS containing $1\ \mu\text{Ci}$ ^3H -5,6,8,9,11,12,14,15-arachidonic acid (specific activity of $64\ \text{Ci mmol}^{-1}$; New England Nuclear, Boston, Massachusetts) for 4 h. At this time the culture medium was removed, the cells washed twice with 5 ml PBS and incubated in 2 ml M199 devoid of serum with or without sonicated zymosan particles (ICN-K+K Labs, Plainview, New York). The stock zymosan preparations were washed and sterilised by boiling three times in PBS. The medium was removed, acidified to 0.03 M with citric acid, authentic standards added, and extraction carried out using the method of Folch. The combined organic phases were dried and chromatographed on SG81 paper (Whatman) with ethyl acetate-acetone-acetic acid (90:10:1). The standard PGE_2 and $\text{PGF}_{2\alpha}$ were visualised using iodine vapour, the corresponding area cut out and the radioactivity corresponding chromatographically with PGE_2 and $\text{PGF}_{2\alpha}$ determined. $n = 3 \pm \text{s.d.}$ Open columns, control cultures; hatched cultures exposed to zymosan $50\ \mu\text{g}$ per culture. Left two columns in each set of four, PGE_2 ; right two, $6\text{KF}_{1\alpha}$.

Macrophages synthesise and release prostaglandins in response to inflammatory stimuli

WHEN macrophages encounter inflammatory stimuli either *in vivo* or *in vitro*, they respond by releasing a number of products which may account for the central role that this cell has in chronic inflammatory diseases¹. These products include hydrolytic enzymes active at neutral² or acidic pH (ref. 1), components of both the classical³ and alternate pathway⁴ of complement, factors modulating responses of lymphocytes to antigens and mitogens⁵, and factor(s) influencing the proliferation⁶ and synthesis of collagen⁷ by fibroblasts. We now show

PGD₂ and PGA₂. When, however, such cultures are exposed to an inflammatory agent, zymosan, they synthesise and release large amounts of ³H-PGs in a time-dependent manner. Significant increases in synthesis and release are observed as early as 30 min after addition of zymosan, continuing thereafter for at least 24 h (Fig. 1). In the separation system described in Fig. 1, two major peaks of radioactivity were found. The first of these peaks had the same mobility as authentic PGE₂. This identification is supported also by radioimmunoassay and by its conversion to PGB₂ on base hydrolysis (R.J.B. *et al.*, unpublished). The second of these peaks had the same mobility as PGF_{2α} and 6-keto PGF_{1α} (6KF_{1α}) which are not separable in this system. Using a second chromatographic system, ethyl acetate-acetic acid, 99:1, which separates these two components, the second radioactive peak has, however, been tentatively identified as 6KF_{1α}. ³H-thromboxane B₂ (TXB₂) does not seem to be a major product of ³H-arachidonic acid from unstimulated macrophages exposed to zymosan.

Macrophages from mice stimulated with thioglycollate broth—a sterile inflammatory agent—have a greatly diminished capacity for synthesising and releasing ³H-PGs in response to increasing amounts of zymosan, compared with cells from unstimulated mice, although both cell populations incorporated approximately the same amount of ³H-arachidonic acid (Fig. 2). At the lowest dose of zymosan tested (10 μg per plate), unstimulated cells showed a fivefold increase in ³H-PGE₂ and a 2.5-fold increase in ³H-6KF_{1α} release. At 200 μg ml⁻¹ zymosan, unstimulated cells showed a 24-fold increase in ³H-PGE₂ and a 15-fold increase in ³H-6KF_{1α} release. On the other hand, cells from thioglycollate-stimulated mice showed only a fivefold increase in ³H-PGE₂ and a 2.5-fold increase in ³H-6KF_{1α} release in response to 200 μg zymosan per plate. In the absence of zymosan, both unstimulated and stimulated cells release similar small amounts of ³H-PGE₂ and ³H-6KF_{1α}. Cells from stimulated or unstimulated mice cultured in the absence or presence of zymosan remained intact as judged by morphological examination and the retention of the cytoplasmic marker enzyme, lactate dehydrogenase.

The synthesis and release of ³H-PGs by unstimulated macrophages exposed to zymosan can be completely inhibited by indomethacin (Fig. 3). The effect of this cyclo-oxygenase inhibitor on ³H-PGs synthesis in this system is a potent one, dose-response experiments showing an ID₅₀ of 2 × 10⁻⁸ M.

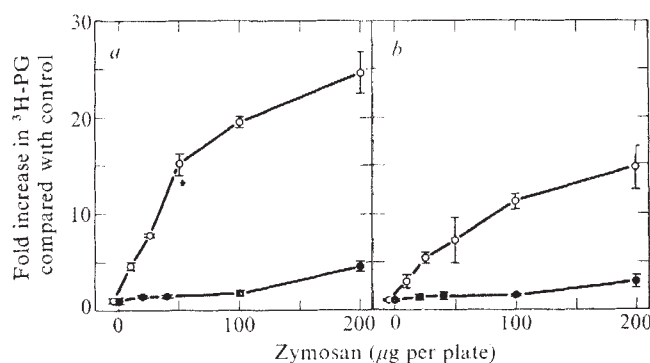


Fig. 2 Thioglycollate-induced macrophages synthesise and release less ³H-PG than unstimulated macrophages in response to zymosan challenge. *a*, PGE₂; *b*, 6KF_{1α}. Unstimulated macrophages and macrophages obtained from mice injected with 2 ml thioglycollate broth (BBL, Cockeysville, Maryland) 4 d previously were cultured and labelled as described in Fig. 1. The radiolabelled cells were then incubated with varying concentrations of zymosan for 4 h. The released radiolabelled PGs were measured as described in Fig. 1. The stimulated cells (●) incorporated 47% of the ³H-arachidonic acid; the unstimulated cells (○) incorporated 57% of the ³H-arachidonic acid. Control cultures of the stimulated cells without zymosan addition contained 365 ± 139 c.p.m. as PGE₂ and 191 ± 12 c.p.m. as 6KF_{1α}; control cultures of the unstimulated cells contained 228 ± 31 c.p.m. as PGE₂ and 102 ± 17 c.p.m. as 6KF_{1α}. *n* = 3 ± s.d.

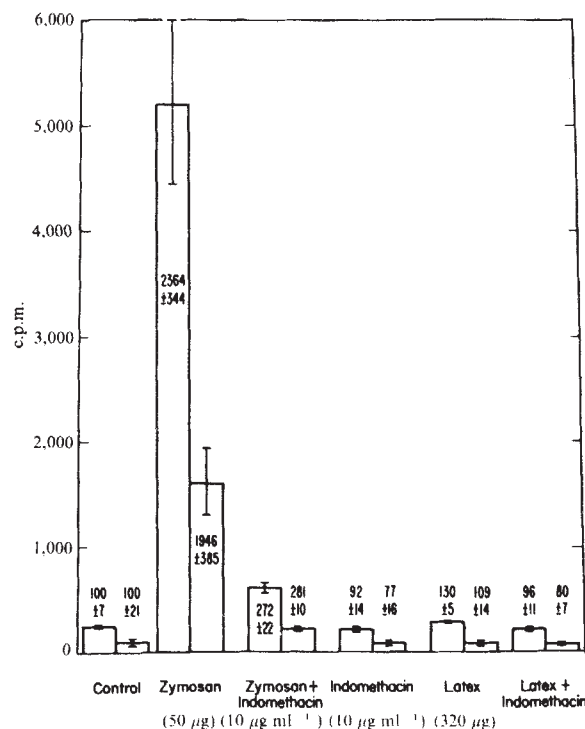


Fig. 3 Zymosan but not latex stimulated ³H-PG synthesis and release from unstimulated macrophages. Unstimulated macrophages were cultured and labelled as described in Fig. 1. The release incubation period was 4 h. Suspensions of latex beads 0.8 μm; (Dow, Indianapolis, Indiana) were sonicated before addition. The numbers in the histogram indicate percentage stimulation over control. *n* = 3 ± s.d. Left-hand column in each pair, PGE₂; right-hand, 6KF_{1α}.

PG synthesis does not always accompany phagocytosis by macrophages. Fig. 3 shows that unstimulated macrophages exposed to a large amount of latex particles do not release ³H-PGs above control levels. Examination of cultures by phase contrast microscopy showed that these particles had been phagocytosed. It is noteworthy that latex lacks the ability to induce chronic inflammation¹⁵.

These findings complement those of Glatt and Brune who have used other stimuli to induce release of PGE₂ by mouse peritoneal macrophages¹⁶. In addition, we have unpublished data showing that macrophages release PGs in response to other inflammatory stimuli such as asbestos and antigen-antibody complexes. Clearly, PGs are now established as products of macrophages responding to inflammatory stimuli. The tentative identification 6KF_{1α} as being a major secretory PG by zymosan-induced macrophages remains to be confirmed. Our data, however, suggest that the potent biologically active precursor, PGI₂, which has been shown to be released by cells of the vascular endothelium¹⁷, may also be released by macrophages. The contribution of PGs released by macrophages to the diverse functions of this cell remains to be elucidated.

We thank Dr E. A. Ham for 6KF_{1α} and TXB₂ standards.

J. L. HUMES
R. J. BONNEY
L. PELUS
M. E. DAHLGREN
S. J. SADOWSKI
F. A. KUEHL, JR
P. DAVIES

Merck Institute for Therapeutic Research,
Rahway, New Jersey 07065

Received 8 June; accepted 5 July 1977.

¹ Davies, P. & Allison, A. C. *Immunobiology of the Macrophage* (ed. Nelson, D. S.) 427-461 (Academic, New York, 1976).

- ² Werb, Z. & Dingle, J. T. *Lysosomes in Biology and Pathology* 5 (eds Dingle, J. T. & Dean, R. T.) 127-156 (North-Holland, Amsterdam, 1976).
- ³ Colten, H. R. & Einstein, L. P., *Transplant. Rev.* 32, 3-11 (1976).
- ⁴ Bentley, C., Bitter-Suermann, D., Hadding, V. & Brade, V. *Eur. J. Immun.* 6, 393-401 (1976).
- ⁵ Waksman, B. H. & Namba, Y. *Cell. Immun.* 21, 161-176 (1976).
- ⁶ Leibovich, S. J. & Ross, R. *Am. J. Path.* 84, 501-513 (1970).
- ⁷ Lewis, D. M. & Burrell, R. *Br. J. ind. Med.* 33, 25-28 (1976).
- ⁸ Myatt, L., Bray, M. A., Gordon, D. A. & Morley, J. *Nature* 257, 227-228 (1975).
- ⁹ Gordon, D., Bray, M. A. & Morley, J. *Nature* 262, 401-402 (1976).
- ¹⁰ Flower, R. J. & Blackwell, J. G. *Biochem. Pharmac.* 25, 285-291 (1976).
- ¹¹ Isaksson, P. C., Raz, A. & Needleman, P. *Prostaglandins* 12, 739-748 (1976).
- ¹² Hong, S. L. & Levine, L. *Proc. natn. Acad. Sci. U.S.A.* 73, 1730-1734 (1976).
- ¹³ Bills, T. K., Smith, J. B. & Silver, M. J. *Biochim. biophys. Acta* 424, 303-314 (1976).
- ¹⁴ Mason, R. J., Stossel, T. P. & Vaughan, M. *J. clin. Invest.* 51, 2399-2407 (1972).
- ¹⁵ Schorlemmer, H.-U., Davies, P., Nylon, W., Gugig, M. & Allison, A. C. *Br. J. exp. Path.* (in the press).
- ¹⁶ Glatt, M. & Brune, K. *Proceedings of Future Trends of Inflammation III* (MTP, Lancaster, in the press).
- ¹⁷ Moncada, S., Gryglewski, R., Bunting, S. & Vane, J. R. *Nature* 263, 663-665 (1976).

Primary *in vitro* sensitisation of human T cells

METHODS for the *in vitro* development of primary immune responses have been crucial for the understanding of mechanisms of cooperation between T and B cells and between T cells and macrophages¹⁻³. Few efforts to date have been reported for primary *in vitro* sensitisation of human lymphocytes, most of which have involved sensitisation to allo- or xeno-antigens⁴⁻⁶ or plaque-forming responses to sheep red blood cells (SRBC)^{7,8}. Development of killer T cells against HLA-identical⁹, or autochthonous tumour cells^{10,11} have usually required coculture with third party allogeneic cells for development of cytotoxicity. Given the ethical constraints on *in vivo* studies of human T-cell function, and noting the development for the first time of a system for the sensitisation of guinea pig T cells *in vitro* to a variety of antigens¹², we were encouraged to develop an *in vitro* system for the priming and boosting of human T cells to well defined haptenic determinants without the requirement for a third party cell.

We chose trinitrophenol (TNP) as antigen for two reasons: in our hands it elicits the best response; and second, animal studies show that when recognised in conjunction with Ia antigens on macrophages, TNP can elicit helper-type T cells¹², and when recognised simultaneously or as an antigenic complex with H-2 K and D antigen can generate killer T cells¹³. The hapten TNP, therefore, has the potential in the human system to serve as a model for primary *in vitro* sensitisation and recognition of altered self.

Tonsil or peripheral blood cells were divided into two portions (Fig. 1). The responder cell population was purified by nylon wool column fractionation; the stimulator population, unfractionated, was treated with mitomycin C or X-ray irradiation and either conjugated with TNBS (trinitrobenzene sulphonate) or left unconjugated as control. Optimum culture conditions required 5×10^6 responder cells plus $1-5 \times 10^6$ stimulator cells in 2 ml of RPMI-1640 containing 10% AB serum and antibiotics. Cultures were fed twice during the 7-d priming. Boosting was done in microtitre plates on day 7 using autochthonous booster cells prepared exactly as were the stimulator cells. Boosting of peripheral blood cells was done with freshly drawn blood; boosting of tonsil cells was done with cells that had been cryopreserved. Cultures did not require 2-mercaptoethanol or rocking (forward and backing tilting during 37 °C incubation). Responses were measured by incorporation of labelled thymidine (³H-TdR), the virus plaque assay and cytotoxicity.

Figure 2 indicates the kinetics of proliferation in the priming and boosting cultures. Nylon wool purified cells alone showed very little proliferation during culture. The addition of mitomycin-treated TNP-coupled or uncoupled cells caused measurable proliferation between days 3 and 5. Optimum boosting conditions required cells primed for at least six and preferably seven days, when background

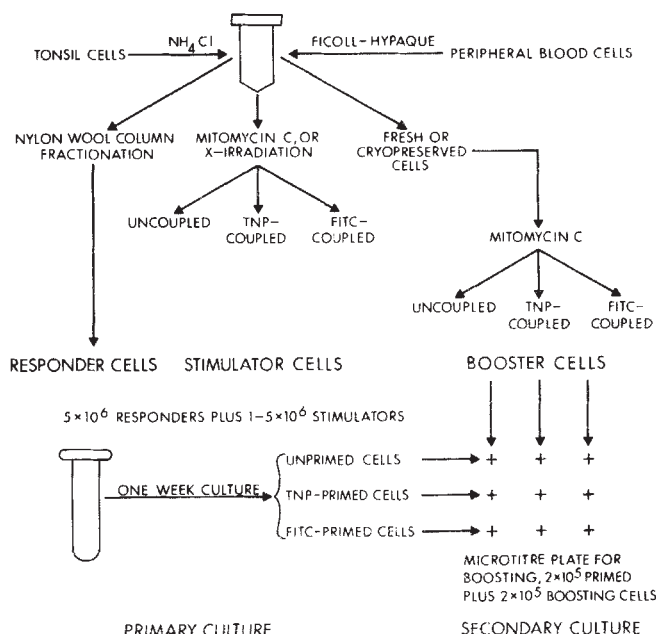


Fig. 1 Schematic representation of the procedure for priming and boosting of T cells to hapten-coupled autochthonous cells. Tonsil cells, after NH_4Cl lysis of red cells or peripheral blood mononuclear cells after Ficol-Hypaque density centrifugation, were divided into two portions. The first was passed over nylon wool columns for preparation of T cells¹⁴. This gave a population containing fewer than 2%-3% Ig+ cells by immunofluorescence. The second unfractionated population was treated with mitomycin C ($50 \mu\text{g ml}^{-1}$, 37 °C, 30 min) or X-ray irradiation (500 rad), and hapten-conjugated or left unconjugated as control. A portion of tonsil cells was cryopreserved in 10% dimethyl sulphoxide-20% foetal calf serum in RPMI under liquid nitrogen until needed for the secondary (boosting) culture. TNP-coupling with 10-mM TNBS in phosphate-buffered saline (PBS) pH 7.4 was done according to Shearer¹⁵, and FITC-coupling was carried out with $100 \mu\text{g}$ FITC per 10^8 cells per ml in PBS pH 9.0 (ref. 16). Primary cultures were set up in 16×125 -mm tissue culture tubes containing 5×10^6 responder and an equal number of stimulator cells in 2 ml of RPMI 1640 containing 10% AB serum, 8 mg per 100 ml gentamycin, 40 units ml^{-1} mycostatin, $100 \mu\text{g ml}^{-1}$ streptomycin and extra glutamine, 2 mM. Primary cultures were fed twice, on days 2 and 5, and kept in a humidified atmosphere of 5% CO_2 , 95% air. Secondary boosting cultures were set up on day 7 with 2×10^5 viable primed cells plus 2×10^5 boosting cells per 0.2 ml in microtitre plates. Boosting cells were prepared as described for stimulator cells using thawed unfractionated tonsil cells or freshly drawn and prepared peripheral blood mononuclear cells. At 16 h before cell collection $1 \mu\text{Ci}$ ³H-TdR (specific activity 3 Ci mmol^{-1}) was added to each well. Cultures were collected on glass fibre filters, placed in scintillant (NEN 950A) and counted in a Beckman liquid scintillation counter. All determinations were done in triplicate.

proliferation had largely ceased. Boosting of TNP-primed cells with TNP-coupled autochthonous cells resulted in a marked and rapid proliferation of the primed cells between 24 and 96 h after boosting; unprimed cells showed no response (Fig. 2).

The boosted response is antigen specific, as shown in Table 1. T cells from each tonsil were primed to either TNP-cells or FITC-cells. After 1 week both cultures were boosted with both antigens. TNP-primed cells responded to TNP-cells and not to FITC cells, whereas FITC-primed cells responded to FITC cells and not to TNP cells. In 14 experiments, TNP-primed cells gave a mean value above background of $14,765 \pm 3,199$ c.p.m. and a mean stimulation index of 7.0 ± 1.6 when boosted with TNP cells. In five experiments, the response of the FITC-primed cells was always less than the response of TNP-primed cells; the mean incremental ³H-TdR incorporation was $3,743 \pm 2,330$ c.p.m. and the stimulation index was 3.18 ± 1.1 . Ultraviolet light microscopy revealed that the stimulating and boosting cells were coupled with FITC at the

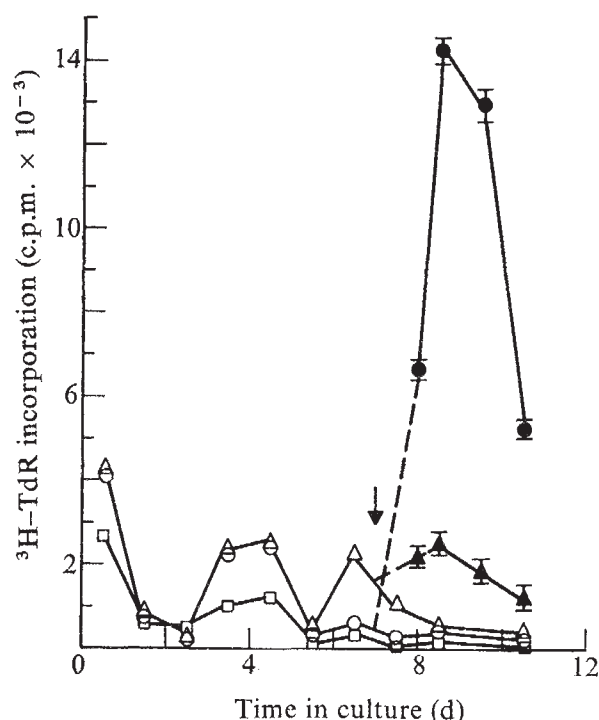


Fig. 2 Time course of incorporation of ^3H -TdR in primary (sensitisation) cultures of 5×10^6 nylon wool cells alone ($\square$), 5×10^6 nylon wool purified cells plus 5×10^6 uncoupled stimulator cells (control) ($\triangle$), and after TNP-boosting ($\blacktriangle$), and 5×10^6 nylon wool purified cells plus 5×10^6 TNP-coupled autochthonous cells ($\circ$), and after TNP-boosting ($\bullet$). The TNP-primed and unprimed cultures were each boosted on day 7 (arrow) with TNP-coupled autochthonous cells, as indicated. S.e.m. is indicated for boosted cultures; for priming cultures, standard errors were always less than 20%.

initiation of culture, and their viability at this time was comparable with that of TNP-coupled cells, that is, greater than 90%.

The response of peripheral blood cells to TNP was usually less than the response given by tonsil T cells (Table 1). In four experiments, three individuals responded (Table 1, and data not shown) while one was completely refractory. Whether this is as a result of individual differences in responder, suppressor or antigen presenting cells types has yet to be determined. To date, we have not been able to achieve a significant *in vitro* response of peripheral blood cells to FITC.

Cultures of T cells plus mitomycin-C treated autochthonous uncoupled cells usually showed a low proliferative response during the priming culture (Fig. 2), as did the T cells cultured together with TNP-coupled cells. When such unprimed control cells were 'boosted'

with uncoupled stimulator cells, the incorporation of ^3H -TdR increased from 2- to 12-fold as compared with their proliferative response when cultured alone (Fig. 2 and Table 1). This seems most consistent with the experiments of Kuntz *et al.*¹⁷, who were able to demonstrate, in humans, a response of purified T cells to autochthonous B and K cells peaking about day 6, though no attempt was made to boost these cells.

The response of primed cells to concanavalin A (con A) was included in all experiments to serve as an internal reference for the relative magnitude of the boost. The response to TNP-cells was in the range of 50% of the con A response. (Table 1).

Viable stimulating cells were required for effective sensitisation. Stimulator cells treated at 56°C for 30 min functioned poorly compared with viable stimulator cells; the response to boosting was only one sixth that of cultures primed with viable cells (data not shown).

We have attempted to estimate the number of antigen-reactive T cells in the boosted cultures using the virus plaque assay¹⁸. Table 2 shows the results of these experiments. The background response was low in all three controls, allowing the detection in specifically boosted cells of approximately one cell per thousand plated. This is a sevenfold increase over primed cells not boosted, and a 50-fold increase over unprimed cells boosted with TNP-cells. These data suggest that the initial number of TNP-reactive cells at day 2 of priming must be relatively low, certainly less than 20 per 10^6 cells. But, one cell in 10^3 in boosted cultures is considerably less than 10–60 per 10^3 cells activated by con A or allogeneic cells^{19,20}, although about 10-fold greater than the number of pre-killer cells activated to TNP in a recently reported murine syngeneic cytotoxicity assay²¹. This may reflect the existence of fewer potentially hapten or self-reactive than allo-reactive clones.

We have attempted to detect a preference on the part of the primed cell for restimulation with TNP coupled to autochthonous cells relative to allogeneic cells. To obviate the problem of allogeneic stimulation, we have taken advantage of the finding that significant stimulation of ^3H -thymidine incorporation can be obtained 24 h after boosting (Fig. 2) when little or no MLR is seen ($\text{SI} < 2.0$). In three experiments, a mean increase of $4,122 \pm 764$ c.p.m. above background was obtained after boosting with TNP-coupled autochthonous (priming) cells, while boosting with TNP-coupled allogeneic cells resulted in a decrease of 27 ± 308 c.p.m. As has been demonstrated in animal models^{22,25}, these results indicate that there is likely to be a restriction on the restimulation of primed T cells to antigen presentation on the priming cell type. Taken together with the requirement for a viable cell for effective antigen presentation, it seems likely that in this system, T cells recognise hapten in conjunction with

Table 1 Response of *in vitro* primed tonsil and peripheral blood T cells to hapten-coupled autochthonous cells

Expt no.	T cells from:	Primed to:	Boosting cell type added, day 3 response (c.p.m.)								FITC cells	Δ c.p.m.	SI	Con A
			None	Uncoupled	TNP cells	Δ c.p.m.	SI							
1	Tonsil	Uncoupled	637 $\pm$ 86*	7,481 $\pm$ 388	9,619 $\pm$ 637	2,138	1.0	12,213 $\pm$ 544	4,732	1.0	19,932 $\pm$ 905			
		TNP	868 $\pm$ 174	10,514 $\pm$ 1,094	39,277 $\pm$ 69	28,763	13.5	14,605 $\pm$ 992	4,091	0.9	19,639 $\pm$ 31			
		FITC	824 $\pm$ 131	8,424 $\pm$ 585	7,321 $\pm$ 1,552	-103	-0.1	20,924 $\pm$ 1,011	12,500	2.6	17,547 $\pm$ 925			
2	Tonsil	Uncoupled	275 $\pm$ 115	246 $\pm$ 38	997 $\pm$ 262	751	1.0	1,165 $\pm$ 245	919	1.0	70,553 $\pm$ 1,856			
		TNP	240 $\pm$ 59	262 $\pm$ 50	6,583 $\pm$ 1,472	6,321	8.4	1,251 $\pm$ 249	989	1.1	65,438 $\pm$ 1,717			
		FITC	216 $\pm$ 65	243 $\pm$ 49	720 $\pm$ 68	477	0.6	2,907 $\pm$ 907	2,664	2.9	67,096 $\pm$ 2,791			
3	Blood	Uncoupled	182 $\pm$ 38	1,334 $\pm$ 204	3,537 $\pm$ 445	2,203	1.0				10,849 $\pm$ 1,269			
		TNP	534 $\pm$ 83	2,727 $\pm$ 86	6,187 $\pm$ 953	3,460	1.6				14,809 $\pm$ 1,393			
4	Blood	Uncoupled†	851 $\pm$ 56	2,543 $\pm$ 88	7,024 $\pm$ 265	4,481	1.0				69,296 $\pm$ 1,102			
		TNP	2,237 $\pm$ 267	8,220 $\pm$ 1,020	33,851 $\pm$ 2,081	25,631	5.7				69,860 $\pm$ 2,100			

*Arithmetic means $\pm$ s.e.m.

†Stimulators in this experiment were irradiated with 500 rad.

SI=Stimulation index

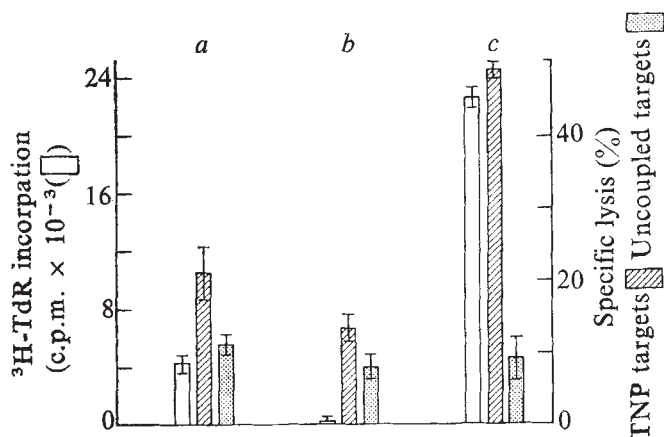


Fig. 3 Virus PFU were determined as for Table 2. *a*, 0.7; *b*, 0.1 and *c*, 1.9 viral plaque-forming cells per 10^5 cells; *a*, TNP-boostered, not primed; *b*, TNP-primed, not boostered; *c*, TNP-primed and boostered. ^3H -TdR incorporation was determined as for Table 1. Cytotoxicity assay was performed in triplicate in round bottom microtitre wells containing 3×10^5 effector cells and 3×10^3 ^{51}Cr labelled targets in 0.2 ml. For preparation of target cells $15 \mu\text{l}$ of 1:100 diluted phytohaemagglutinin P (DIFCO) was added to 2×10^6 tonsil cells in 2 ml of medium 3 d before assay. On day of assay, targets were labelled with $\text{Na}^{51}\text{CrO}_4$ (Amersham/Searle, 1 mCi ml^{-1}), $250 \mu\text{Ci}$ in 0.4 ml per 15×10^6 cells for 2 h at 37°C . Cells were washed three times and either left uncoupled or coupled with TNBS¹⁵. Effectors plus targets were incubated at 37°C for 16 h after which plates were centrifuged and 0.1 ml was collected and counted in an LKB model 80000 gamma counter. Spontaneous release values for TNP-coupled and uncoupled targets were the same, 47% of total releasable counts as determined by three cycles of freezing and thawing; all assays were performed on the tenth day of culture (day 3 after boosting).

membrane determinants. In the mouse system, Forman has shown that TNP couples directly to H-2 antigens on the cell surface, and that they, most likely, are the site of recognition by prekiller T cells^{26,27}. It will be of obvious interest to determine whether HLA antigens are involved in the recognition of TNP in the present system, and if so, whether there is any consistent restriction like that seen with cytotoxic T cells generated against Y antigen²⁸. On the other hand, the ability of primed T cells to discriminate effectively between TNP and FITC is compatible with the view that some T cells may primarily recognise hapten, a view supported by several studies in experimental animals²⁹⁻³¹.

Table 2 Enumeration of activated T cells primed and boosted *in vitro* by virus plaque assay

Boosting cell	Assay	Tonsil T Cells	
		Unprimed	TNP-primed
Uncoupled cells	^3H -TdR incorporation	20,624 $\pm$ 1,171	6,503 $\pm$ 875
	V-PFC/ 10^3 Cells	0.045 $\pm$ 0.001	0.15 $\pm$ 0.01
TNP-coupled cells	^3H -TdR incorporation	12,481 $\pm$ 414	49,153 $\pm$ 1,174
	V-PFC/ 10^3 Cells	0.02 $\pm$ 0.002	1.02 $\pm$ 0.23

Assay for virus plaques was performed 40 h after boosting. Values are arithmetic means $\pm$ s.e.m. V-PFC, virus plaque-forming cells.

Attempts were made to ascertain whether specific cytotoxic T lymphocytes (CTL) could be generated. The data presented in Fig. 3 indicate that CTL capable of killing TNP-coupled targets are generated from naive human T cells after *in vitro* priming and boosting with TNP-coupled autochthonous cells. No CTL were detectable in cultures that were primed but not boosted, or not primed and

'boosted'. In contrast with results obtained in the mouse system¹⁵, CTL were not found in primary cultures, but only in restimulated cultures. It is unclear whether CTL in man are developed on first exposure to antigen but at undetectable levels, or whether a further differentiative step requiring restimulation is necessary. It will also be of interest to clarify the nature of the antigen-presenting cell(s) in this system.

We thank Ethan Shevach for helpful discussions. This work was supported by USPHS grants AI 09807, AI 10702 and a grant from The Jane Coffin Childs Memorial Fund for Medical Research. W. F. is a Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

WALTER NEWMAN
GERALD L. STONER*
BARRY R. BLOOM

Department of Microbiology and Immunology,
Albert Einstein College of Medicine,
Bronx, New York 10461

Received 2 May; accepted 18 July 1977.

*Present address: Armauer Hansen Research Institute, PO Box 1005, Addis Ababa, Ethiopia.

- Waldron, J. A., Horn, R. T. & Rosenthal, A. S. *J. Immun.* **111**, 58-64 (1973).
- Shortman, K. & Palmer, J. *Cell. Immun.* **2**, 399-410 (1971).
- Cheers, C., Bretnier, J., Little, M. & Miller, J. F. A. P. *Nature new Biol.* **232**, 248-250 (1971).
- Sheehy, M. J., Sondel, P. M., Bach, M. L., Wank, R. & Bach, F. H. *Science* **188**, 1308-1310 (1975).
- Lindahl, K. F. & Bach, F. H. *J. exp. Med.* **144**, 305-318 (1976).
- Long, M. A., Handwerker, B. S., Amos, B. & Yunis, E. J. *J. Immun.* **117**, 2092-2099 (1976).
- Luzzati, A. L., Taussig, M. J., Meo, T. & Pernis, B. J. *exp. Med.* **144**, 573-585 (1976).
- Dosch, H. & Gelfand, E. W. *J. Immun.* **118**, 302-308 (1977).
- Sondel, P. M., O'Brien, C., Porter, L., Schlossman, S. & Chess, L. J. *Immun.* **117**, 2197-2203 (1976).
- Zarling, J. M., Raich, P. C., McKeeough, M. & Bach, F. H. *Nature* **262**, 691-693 (1976).
- Golub, S. H. *Cell. Immun.* **28**, 379-389 (1977).
- Thomas, D. W. & Shevach, E. M. *J. exp. Med.* **144**, 1263-1273 (1976).
- Shearer, G. M., Rehn, T. G. & Garbarino, C. A. *J. exp. Med.* **141**, 1348-1364 (1975).
- Greaves, M. F., Janossy, G. & Curtis, P. in *In Vitro Methods in Cell-Mediated and Tumour Immunity* (eds Bloom, B. R. & David, J. R.) 217-227 (Academic, New York, 1976).
- Shearer, G. M. *Eur. J. Immun.* **4**, 527-533 (1974).
- Starzinski-Powitz, A., Pfizenmaier, K., Röllinghoff, M. & Wagner, H. *Eur. J. Immun.* **6**, 799-805 (1976).
- Kuntz, M. M., Innes, J. B. & Weksler, M. E. *J. exp. Med.* **143**, 1042-1054 (1976).
- Sutcliffe, S., Kadish, A., Stoner, G. & Bloom, B. R. in *In Vitro Methods in Cell-Mediated and Tumour Immunity* (eds Bloom, B. R. & David, J. R.) 319-331 (Academic, New York, 1976).
- Jimenez, L., Bloom, B. R., Blume, M. R. & Oettingen, H. F. *J. exp. Med.* **133**, 740-751 (1971).
- Bloom, B. R. et al. *Cold Spring Harb. Symp. quant. Biol.* **41**, 73-83 (1976).
- Lindahl, K. F. & Wilson, D. B. *J. exp. Med.* **145**, 508-522 (1977).
- Pierce, C. W., Kapp, J. A. & Benacerraf, B. *J. exp. Med.* **144**, 371-381 (1976).
- Zinkernagel, R. *Nature* **261**, 139-141 (1976).
- von Boehmer, H. & Haas, W. *Nature* **261**, 141-142 (1976).
- Pfizenmaier, K., Starzinski-Powitz, A., Rodt, H., Röllinghoff, M. & Wagner, H. *J. exp. Med.* **143**, 999-1004 (1976).
- Forman, J., Vitetta, E. S., Hart, D. & Klein, J. *J. Immun.* **118**, 797-802 (1977).
- Forman, J., Vitetta, E. S. & Hart, D. *J. Immun.* **118**, 803-808 (1977).
- Goulmy, E., Termijtelen, A., Bradley, B. A. & van Rood, J. J. *Nature* **266**, 544-545 (1977).
- Alkan, S. S., Williams, E. B., Nitecki, D. E. & Goodman, J. W. *J. exp. Med.* **135**, 1228-1246 (1972).
- Janeway, C. A., Cohen, B. E., Ben-Sasson, S. Z. & Paul, W. E. *J. exp. Med.* **141**, 42-55 (1975).
- Cosenza, H., Julius, M. H. & Augustin, A. A. *Immun. Rev.* **34**, 3-33 (1977).

Genetic non-responsiveness of murine fibroblasts to bacterial endotoxin

LETHAL infections with Gram-negative bacteria are thought to be due in large part to the toxicity of cell wall lipopolysaccharide (endotoxin). The mechanisms of action of lipopolysaccharide (LPS) have recently been clarified by *in vitro* studies which have helped explain many of the pathophysiological sequelae of endotoxin exposure^{1,2}. C3H/HeJ mice, which are genetically resistant to many of the effects of endotoxin, have proved a valuable model for the systematic investigation of endotoxin-mediated phenomena³⁻⁵. Bacterial endotoxins are able to interact

in vitro with a variety of cells and serum factors^{1,2,6} and it is known that LPS acts on murine B lymphocytes as a polyclonal activator for both mitogenesis and immunoglobulin synthesis⁷. This function has been found to be deficient in the C3H/HeJ mouse^{3,4}. Also, endotoxins activate macrophages⁸⁻¹⁰, and this function also seems to be deficient in C3H/HeJ mice⁵ (Ryan, J. L., Glode, L. M. and Nathan, C. F., unpublished observations). To determine if the C3H/HeJ genetic defect in responsiveness to endotoxin is more general, we have investigated the ability of bacterial endotoxin to stimulate C3H/HeJ embryonic fibroblasts. Fibroblasts were chosen because it has recently been shown that they are endotoxin sensitive cells¹¹.

To measure responsiveness of fibroblasts to LPS, we have assayed their rate of glucose utilisation in culture. This has previously been shown to be a sensitive assay for LPS-induced activation of spleen cells and peritoneal cells (Ryan, J. L. *et al.*, unpublished observations). Fibroblasts were isolated from 12–14 d C3H/HeJ and C3H/HeN embryos by removing the head, extremities, and liver, and mincing the remaining tissue with forceps before incubating with BSS and trypsin–EDTA for 10 min each. The tissue was further incubated with trypsin–EDTA for 20 min and the resulting cell suspension filtered through nylon gauze. These filtered cells were cultured at a density of 10^6 per 10 ml in 100-mm plastic tissue culture dishes using Dulbecco's modified MEM with 10% FCS. Cultures were collected with trypsin and transferred to new plates every 5–7 d.

To measure glucose utilisation, 5×10^4 fibroblasts which had been passed at least once were plated in flat-bottomed microtitre plates. This number of embryonic fibroblasts forms almost a complete monolayer in the wells once the cells have spread. Two types of LPS were used to stimulate the cultures. Both were derived from *Escherichia coli* K235, one by phenol extraction¹² and the other by butanol extraction¹³. It has previously been shown that butanol-extracted K235 LPS contains protein tightly bound to the lipid A moiety which activates C3H/HeJ spleen cells. This protein is not present in phenol-extracted K235 LPS which is thus unable to activate C3H/HeJ lymphocytes while having the ability to activate C3H/HeN lymphocytes normally^{14,15}.

The rate of glucose utilisation by C3H/HeJ and C3H/HeN fibroblast cultures stimulated by K235 LPS is shown in Table 1. These data show that glucose utilisation can be used to measure responsiveness of murine fibroblasts to LPS and that C3H/HeJ fibroblasts are not

Table 1 Rate of glucose utilisation by C3H/HeJ and C3H/HeN fibroblast cultures stimulated by K235 LPS

LPS	C3H/HeN (nmol glucose used per 24 h)	C3H/HeJ (nmol glucose used per 24 h)
None	384 ± 8.0	148 ± 2.0
Phenol K235	502 ± 18	144 ± 4.0
Butanol K235	574 ± 32	264 ± 2.0

The glucose content of 10- μ l aliquots of culture supernatant was determined enzymatically during a 2–3-d culture period. Each value represents the total glucose used over an average 24-h period by a culture of 5×10^4 fibroblasts. Values are means $\pm$ s.e.m. of triplicate cultures. The amount of glucose in each aliquot was determined by adding 0.9 ml of the enzyme mixture to each 10- μ l aliquot, mixing vigorously and reading the A_{440} after a 2-min equilibration time. The enzyme mixture was composed of 0.7 ml buffer (0.1 M Tris, 0.064 M NaCl, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 7.5) (ref. 21), 0.1 ml 20 mM ATP, 0.1 ml 5 mM NADP and 5 μ l of a mixture of hexokinase and glucose 6-phosphate dehydrogenase (250 U ml⁻¹ hexokinase). The mixed enzymes were obtained from Sigma Chemical (St Louis, Missouri). Both types of LPS were added to a final concentration of 10 μ g ml⁻¹. Cultures were carried out in flat-bottomed microtitre plates using 200 μ l Eagles MEM supplemented with glutamine 1 mM, streptomycin 10 μ g ml⁻¹, penicillin 100 U ml⁻¹, and gentamycin 25 μ g ml⁻¹. LPS was the gift of Dr D. C. Morrison, Scripps Clinic, La Jolla, California.

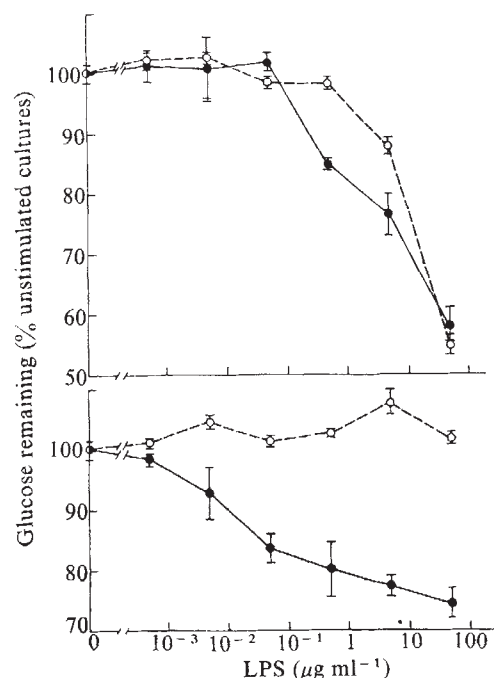


Fig. 1 The upper graph shows the amount of glucose remaining in cultures of C3H/HeN (continuous line) and C3H/HeJ embryonic fibroblasts (dotted line) after stimulation by different concentrations of butanol K235 LPS compared with unstimulated cultures. The lower graph shows similar data for cultures stimulated by phenol K235 LPS. Each point represents the mean of glucose assays from triplicate cultures $\pm$ s.e.m. Culture conditions and glucose assays were as described previously. Measurements on C3H/HeN fibroblasts were made after 32 h of culture, whereas those on C3H/HeJ fibroblasts were made after 66 h of culture, to equate the total glucose utilisation by both cultures at the 50% level. —, C3H/HeJ; — —, C3H/HeN.

responsive to phenol K235 LPS, whereas they are responsive to butanol K235 LPS ($P < 0.01$). In addition, it seems that approximately equal numbers of the C3H/HeJ fibroblasts use glucose at a lower rate than C3H/HeN fibroblasts in both the presence and absence of LPS. The significance of the lower baseline rate of glucose utilisation in C3H/HeJ fibroblasts is not explained, but a similar phenomenon has been observed for C3H/HeJ macrophages (Ryan, J. L. *et al.*, unpublished observations).

A dose-response curve for both types of LPS in C3H/HeJ and C3H/HeN fibroblast cultures is shown in Fig. 1. As seen in the upper graph, both strains respond well to butanol LPS. The lowest dose at which a significant response can be measured is $0.5\text{--}5 \mu\text{g ml}^{-1}$ ($P < 0.01$) LPS for the butanol-extracted material. Activation by phenol K235 LPS is shown in the lower graph. In this case the C3H/HeJ fibroblasts are not stimulated at any of the doses tested ($P < 0.001$), whereas the C3H/HeN fibroblasts are stimulated at a dose of 50 ng ml^{-1} LPS ($P < 0.01$), which is at least 10-fold lower than the concentration of butanol LPS required for activation. This suggests that the protein present in the butanol LPS may in fact be blocking the activity of the lipid A moiety. Modulation of the biological activities of lipid A by protein associated with lipid A has been suggested¹⁶ and these data seem to corroborate those conclusions.

C3H/HeJ non-responsiveness to LPS has been demonstrated in B lymphocytes^{3,4}, macrophages⁵, and now in embryonic fibroblasts. This finding suggests that there is a global cellular defect in the ability of the C3H/HeJ mouse to respond to highly purified LPS. It has also been shown in many laboratories that C3H/HeJ mice are very resistant to the lethal effects of intravenous LPS having an LD_{50} of 3,000 μg , whereas normal mice usually have an LD_{50} of 200–500 μg LPS (ref. 17).

We may conclude that activation of LPS-sensitive cells may have a critical role in mediating the lethal effect of intravenous LPS (ref. 17). It must be recognised, however, that this non-responsiveness of C3H/HeJ mice to LPS does not seem to protect them from death due to infection with Gram-negative bacteria¹⁵⁻¹⁸. Thus, it is evident that there are factors other than LPS which contribute to the toxicity of Gram-negative bacteria. The role of cell wall lipoprotein in mediating toxicity is only beginning to be defined²⁰. Studies using LPS non-responder mice should permit a better understanding of the function of non-LPS cell wall material in producing the pathophysiological effects of endotoxin.

JOHN L. RYAN
KEITH P. W. J. MCADAM

Immunology Branch,
National Cancer Institute,
National Institutes of Health,
Bethesda, Maryland 20014

Received 29 May; accepted 11 July 1977.

- ¹ Morrison, D. C. & Kline, L. F. *J. Immun.* **118**, 362-368 (1977).
- ² Morrison, D. C. & Cochrane, C. J. *exp. Med.* **140**, 797-811 (1974).
- ³ Sultz, B. M. & Nilsson, B. S. *Nature new Biol.* **240**, 198-200 (1974).
- ⁴ Watson, J. & Riblet, R. M. *J. exp. Med.* **140**, 1147-1161 (1974).
- ⁵ Chedid, L. *et al. Infect. Immun.* **13**, 722-727 (1976).
- ⁶ Kabir, S. & Rosenstreich, D. L. *Infect. Immun.* **15**, 156-164 (1977).
- ⁷ Moller, G., Andersson, J. & Sjöberg, O. *Cell. Immun.* **4**, 416-424 (1972).
- ⁸ Wahl, L. M., Wahl, S. M., Mergenhagen, S. E. & Morton, G. R. *Proc. natl. Acad. Sci. U.S.A.* **71**, 3598-3601 (1974).
- ⁹ Weiner, E. & Levanon, D. *Lab. Invest.* **19**, 584-590 (1968).
- ¹⁰ Shilo, M. A. *Rev. Microbiol.* **45**, 255-278 (1959).
- ¹¹ Vaheri, A., Ruoslahti, E., Sarvas, M. & Nurminen, M. *J. exp. Med.* **138**, 1356-1364 (1973).
- ¹² McIntire, F., Sievert, H., Barlow, G., Finley, R. & Lee, A. *Biochemistry* **6**, 2363-2372 (1967).
- ¹³ Morrison, D. C. & Leive, L. *J. biol. Chem.* **250**, 2911-2919 (1975).
- ¹⁴ Morrison, D. C., Betz, S. J. & Jacobs, D. M. *J. exp. Med.* **144**, 840-846 (1976).
- ¹⁵ Sultz, B. M. & Goodman, G. W. *J. exp. Med.* **144**, 821-827 (1976).
- ¹⁶ Betz, S. J. & Morrison, D. C. *Fedn Proc.* **36**, 1234 (1977).
- ¹⁷ Glode, L. M., Mergenhagen, S. E. & Rosenstreich, D. L. *Infect. Immun.* **14**, 626-630 (1976).
- ¹⁸ von Jeney, N., Gunther, E. & Jann, K. *Infect. Immun.* **15**, 26-33 (1977).
- ¹⁹ Rowley, D. J. *Infect. Dis.* **123**, 317-327 (1971).
- ²⁰ Wober, W. & Alaupovic, P. *Eur. J. Biochem.* **19**, 357-367 (1971).
- ²¹ Kinsky, S. C., Haxby, J. A., Zopf, P. A., Alving, C. R. & Kinsky, C. B. *Biochemistry* **8**, 4149-4158 (1969).

Density-dependent inhibition of fibroblast growth is overcome by pure mitogenic factors

CELL density is of interest because it may have a critical role in the regulation of growth, development, and in malignant transformation. In cell culture, crowding slows and eventually stops cell proliferation of a variety of cells maintained on a solid surface¹⁻⁵. This property is usually lost after malignant transformation¹⁻⁵. Two major hypotheses have evolved to explain this phenomenon. The contact inhibition hypothesis holds that specific surface receptors are activated by cell-cell contact and generate a negative signal which halts further growth¹⁻³. The humoral hypothesis proposes that high cell density limits the availability of medium components, particularly of growth factors present in serum^{4,5}. Although much experimental evidence has been adduced in support of the humoral hypothesis, most experiments in which the effect of cell density on growth has been thoroughly investigated were performed in the presence of whole serum as the source of growth factors. The chemical complexity of serum leaves open the possibility that some of its effects may be exerted by molecules that interfere with inhibitory cell-cell contacts. Thus, definitive evidence for a humoral hypothesis in growth regulation in culture is still lacking. To circumvent this objection we have examined the effect of cell density in a model system in which chemically defined molecules rather than whole serum stimulate DNA synthesis and cell division in sparse or crowded cells arrested in G₀/G₁. We report here that density dependent inhibition of fibroblast growth is overcome by pure mitogenic factors.

Epidermal growth factor (EGF) and insulin, added at low concentrations dramatically stimulate DNA synthesis in 3T6 cells maintained in serum-free Dulbecco's modified Eagle's medium (DEM) supplemented with vitamin B₁₂ (ref. 6). To establish whether the response of the cells to constant concentrations of EGF and insulin is dependent on the cell density of the culture, cell suspensions were plated at different densities, arrested in G₀ by limitation of serum and then exposed to factors and ³H-thymidine for 26 h (Fig. 1). The labelling index is optimal at a density of approximately 2 × 10⁴ cells cm⁻² but strongly decreases at higher concentrations where extensive cell contacts take place. Figure 1 also shows that EGF and insulin stimulate DNA synthesis at low densities (0.5 × 10⁴ cells cm⁻²) where only 5% of the available surface is occupied by 3T6 cells and very few cells in close contact are observable microscopically. This renders unlikely the possibility that the pure peptides act by diminishing inhibitory cell-cell contacts. Thus the striking dependence of stimulation on cell density raises the possibility of investigating this phenomenon in chemically defined conditions rather than in the presence of serum.

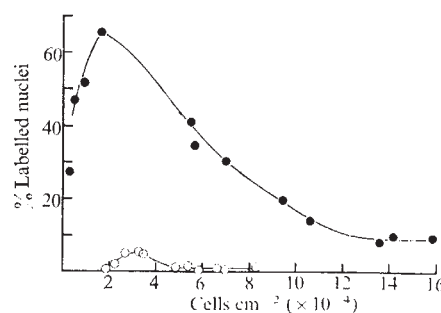


Fig. 1 Effect of cell density on the stimulation of cultures of 3T6 cells into the S-phase by EGF and insulin. Cultures were exposed to 1 ng ml⁻¹ EGF, 100 ng ml⁻¹ insulin, 400 ng ml⁻¹ vitamin B₁₂ and ³H-thymidine (4 µCi ml⁻¹ 0.2 µM) in DEM for 26 h. They were then fixed for autoradiography. Each solid circle represents the mean of two separate determinations (deviation not greater than 5%). Control dishes (B₁₂ containing DEM alone) pooled from a number of experiments are shown by open circles. Stock 3T6 cells were passaged at 3-d intervals in DEM containing 10% foetal calf serum (FCS), 100 U ml⁻¹ penicillin and 100 ng ml⁻¹ streptomycin in a 10% CO₂ atmosphere at 37 °C. In all experiments cell suspensions were plated in Nunc dishes at the appropriate density in 0.5% FCS-DEM, and kept for at least 5 d; they were then washed twice in DEM and left in serum-free medium for 2 d before use. The cell density (abscissa) represents the density at the end of the experiment.

A critical prediction of the humoral hypothesis is that raising the concentrations of the same pure factors that support growth in sparse cultures should overcome the inhibition of growth characteristic of high-density cultures. To test this prediction we determined the dose response for stimulation of DNA synthesis by EGF and insulin in cultures prepared at two different cell densities, sparse and dense (Fig. 2). EGF is more mitogenic in sparse than in dense cultures. Thus EGF, in the absence of insulin stimulates 60% of the cells blocked in G₀ at low density to enter into the S-phase of the cell cycle (Fig. 2a), while it only stimulates 30% of the cells of the denser culture (Fig. 2b). In turn, insulin stimulates DNA synthesis in sparse cultures, even in the absence of EGF and markedly enhances the response to EGF in both sparse and dense cultures. The salient feature of Fig. 2 is that a high concentration of factors is capable of maximally stimulating DNA synthesis in the dense cultures. Essentially similar results were obtained when incubation time was 24 instead of 48 h and also when the total ³H-thymidine incorporated, rather than the labelling index, was measured. The data indicate

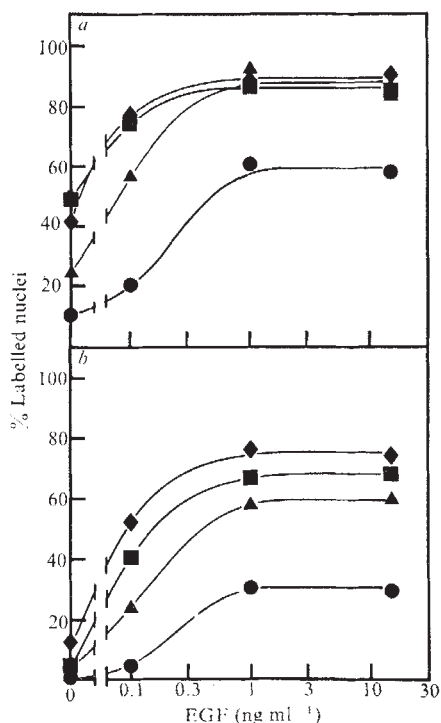


Fig. 2 Dose response to EGF at various insulin concentrations for two different cell densities: *a*, 3.0×10^4 cells cm^{-2} ; *b*, 6.5×10^4 cells cm^{-2} . Insulin concentrations: $\bullet$, zero; $\blacktriangle$, $0.1 \mu\text{g ml}^{-1}$; $\blacksquare$, $1 \mu\text{g ml}^{-1}$; $\blacklozenge$, $10 \mu\text{g ml}^{-1}$. Cultures were exposed to mitogens and ^3H -thymidine for 48 h and fixed for autoradiography.

that high levels of factors overcome the density dependent inhibition of growth.

To further substantiate this conclusion we investigated the effect of cell density on the ability of stimulated cells to complete the cell cycle and divide. Cultures were prepared at two different densities (3.3×10^4 and 17.4×10^4 cells cm^{-2}), arrested in G_0 and then exposed to factors at low and high concentrations for 72 h. At the end of the incubation the number of cells per culture was determined. Table 1 shows that both concentrations of factors stimulate an increase in cell number (70%) in sparse cultures. In contrast, only the combination of factors at high concentrations is able to produce a comparable increase in the crowded cultures. This result, together with the autoradiographic data clearly demonstrates that the growth block imposed by high cell density can be overcome by using appropriate levels of pure factors.

We considered the possibility that the ability of high

Table 1 Effect of cell density on growth

EGF (ng ml $^{-1}$)	Insulin ($\mu\text{g ml}^{-1}$)	Cell density (cells $\text{cm}^{-2} \times 10^{-4}$)	
		Sparse	Dense
—	—	2.5	13.3
1	0.2	5.6	14.8
10	5.0	5.4	24.1

Cells were plated at two densities in 50-mm Nunc dishes and arrested in G_0/G_1 before exposure to factors. Cell densities before exposure were 3.3×10^4 and 14.7×10^4 cells cm^{-2} for sparse and dense cells respectively. Two levels of factors were used. After 72 h incubation, cells were removed by trypsinisation and counted with a Coulter counter. In sparse cultures for each determination six dishes were divided into two groups of three pooled dishes. In dense cultures nine dishes were divided into three groups of three pooled dishes. Three aliquots of each group were counted. Data are mean group values. Group duplicates and triplicates did not vary by more than 15% and in general were below 7.5%.

levels of insulin to potentiate the effect of EGF in crowded cultures may be due to a mechanism of action unrelated to its hormonal effect, such as the mild proteolytic activity reported to be associated with the pure hormone and with the B chain of the molecule⁷. We found several lines of evidence that exclude this possibility in our system: (1) The purified B chain of the insulin molecule does not replace the hormone in stimulating the entry of sparse or dense cultures into S. (2) The B chain, added with sufficient amounts of insulin and EGF to stimulate low density cultures, does not overcome the density block in crowded cells. (3) Ovomucoid ($100 \mu\text{g ml}^{-1}$) and soybean ($50 \mu\text{g ml}^{-1}$) trypsin inhibitors which were reported to suppress the proteolytic activity of insulin⁷, do not prevent the enhancing effect of high concentrations of insulin. Furthermore, we verified that the preparation of insulin used in our studies yields a single band (molecular weight 6,400) when analysed in sodium dodecyl sulphate-polyacrylamide gel electrophoresis. In addition zinc, which is present in the insulin preparation and has been shown to promote growth⁸ is completely inactive when tested at 6×10^{-8} M and 6×10^{-7} M in the presence and absence of factors. All these experiments indicate that in crowded cultures as in sparse ones, the action of insulin is related to its metabolic effects mediated by specific surface receptors.

Our results emphasise that chemically defined growth promoting factors used in *in vitro* systems can overcome the restriction of growth imposed by high cell density. These findings have two interesting implications. First, the use of two pure peptides instead of a complex substance like serum for overcoming the block imposed by high cell density removes a major objection in accepting a humoral rather than a contact hypothesis for regulation of growth. The possibility remains that the pure peptides act by diminishing contact inhibition but this seems unlikely because the factors are effective at low cell densities (Fig. 1). Second, the fact that cells transformed by some RNA tumour viruses have an alteration in the EGF growth regulatory mechanisms⁹, that cultures of BHK cells transformed by SV40 viruses produce growth promoting polypeptides¹⁰ and our finding that defined peptides can release cells from density dependent inhibition of growth suggest that growth factors may have a critical role in modulating normal and abnormal cell proliferation.

KARL MIERZEJEWSKI
ENRIQUE ROZENGURT

Imperial Cancer Research Fund Laboratories,
Lincoln's Inn Fields,
London, UK

Received 26 April; accepted 12 July 1977.

- Dulbecco, R. in *Growth Control in Cell Cultures* (eds Wolstenholme, G. E. W. & Knight, J.) 71 (Churchill Livingstone, Edinburgh and London, 1971).
- Noonan, K. D. & Burger, M. M. *Prog. Surf. Membr. Sci.* 8, 245 (1974).
- Westermarck, B. *Biochem. biophys. Res. Commun.* 69, 304 (1976).
- Holley, R. W. *Nature* 258, 487 (1975).
- Rozengurt, E. in *Eukaryotic Cell Function and Growth* (eds Dumont, J. E., Brown, B. L. & Marshall, N. J.) 711 (Plenum, New York, 1976).
- Mierzejewski, K. & Rozengurt, E. *Biochem. biophys. Res. Commun.* 73, 271 (1976).
- Huang, D. & Cuatrecasas, P. *J. cell. Biol.* 250, 8251 (1975).
- Rubin, H. *Proc. natn. Acad. Sci. U.S.A.* 69, 712 (1972).
- Todaro, G. J. & De Larco, J. E. *Nature* 264, 26 (1976).
- Bourne, H. R. & Rozengurt, E. *Proc. natn. Acad. Sci. U.S.A.* 73, 4555 (1976).

Cultured human breast cancer cells lose selectivity in direct intercellular communication

VARIOUS small molecules, up to 1,600 molecular weight¹, can pass directly between certain animal cells in contact, probably through gap junctions^{2,3}. Such communication

may partly mediate growth control in multicellular organisms and cultured cells, so that disturbances in the normal pattern of communication could be related to malignant growth^{4,5}. The ³H-nucleotide transfer method⁶, based on metabolic cooperation⁷ has shown that communication is not always an all-or-none phenomenon, but may be selective^{8,9}. For example, human mammary epithelial cells from benign tumours or milk do not communicate with human breast fibroblasts (HumF), although either type can communicate well with homologous cells. We have examined the communication between cancer cells from several human breast cancer sources and found that, unlike normal

mammary epithelium, they do not show selectivity in communication. The cancer cells fall into one or other of the two functional groups we have defined as non-selective communicators (communicate with all cells capable of communication) or non-communicators (unable to communicate with homologous or heterologous cells) suggesting that the change to malignancy involves some change in the pattern of cell communication.

The breast cancer cells were either established lines derived from malignant pleural effusions, or primary cultures obtained by the 'spillage' technique¹⁰, from surgical specimens¹¹. Two established lines, MCF-7 (ref. 12) and MDA-157 (ref. 13), and three primary cultures, T162, T231 and T235, were examined. The putative cancer cells which are obtained from primary and metastatic carcinomas by the spillage method, we have called HumE¹ (ref. 11). These cells have a low labelling index (less than 2% in 48 h), but metabolise and can incorporate ³H-uridine into their nucleotide pool, and hence may be used as donors.

The transfer of ³H-uridine nucleotides from prelabelled donor cells was tested using as recipients representatives of each functional group, that is, non-communicators (L929), non-selective communicators (calf lens), and selective communicators (HumF) unable to communicate with normal HumE cells. Transfer of nucleotide was quantitated by grain counts from autoradiographs as described in Fig. 1, which summarises the results and presents data obtained previously using HumE cells as donors. The cell line MCF-7 and cells from the primary carcinoma T231 show a complete loss of ability to communicate with any of the recipients tested. Cell line MDA-157 and cells from T162 and T235, however, show a loss of selectivity in communication and donate nucleotide not only to calf lens but also to human mammary fibroblasts. Further experiments (data not shown) with the two established lines confirmed that MCF-7 did not communicate with itself whereas MDA-157 retained this ability.

The main problem in interpreting this data is that HumE¹ cells from primary carcinomas have not been fully characterised as the actual cancer cells. They have, however, been shown to be epithelial in ultrastructure¹¹, and are the only cells other than human mammary epithelium (HumE), and human mammary fibroblasts (HumF), that have been obtained from primary or secondary breast cancers in this laboratory. HumE¹ cells have never been derived from non-malignant breast tissue or breast secretions, and it is therefore most likely that they do represent the malignant cells of the carcinoma.

This being so, the data presented here show that malignant change may be associated not only with a loss of ability to communicate, but also with a loss of specificity in communication. Other workers have shown that whereas some cancer cells show a reduced ability to communicate directly with other cells^{4,5}, some are good communicators¹⁴. Our results suggest that in both cases a change in the pattern of communication might have occurred. Such changes could be selectively advantageous for the metastasis of cancer cells, enabling them as non-communicators to ignore inhibitory signals from the other epithelial cells, or, as non-selective communicators to receive stimulatory messages from abnormal sources such as fibroblasts.

We thank Dr M. G. P. Stoker for his valuable comments.

IAN S. FENTIMAN
JOYCE TAYLOR-PAPADIMITRIOU

Imperial Cancer Research Fund,
Lincoln's Inn Fields,
London, UK

Received 27 May; accepted 19 July 1977.

¹ Simpson, I., Rose, B. & Loewenstein, W. R. *Science* **195**, 294 (1977).

⁴ Gilula, N. B., Reeves, O. R. & Steinbach, A. *Nature* **235**, 262 (1972).

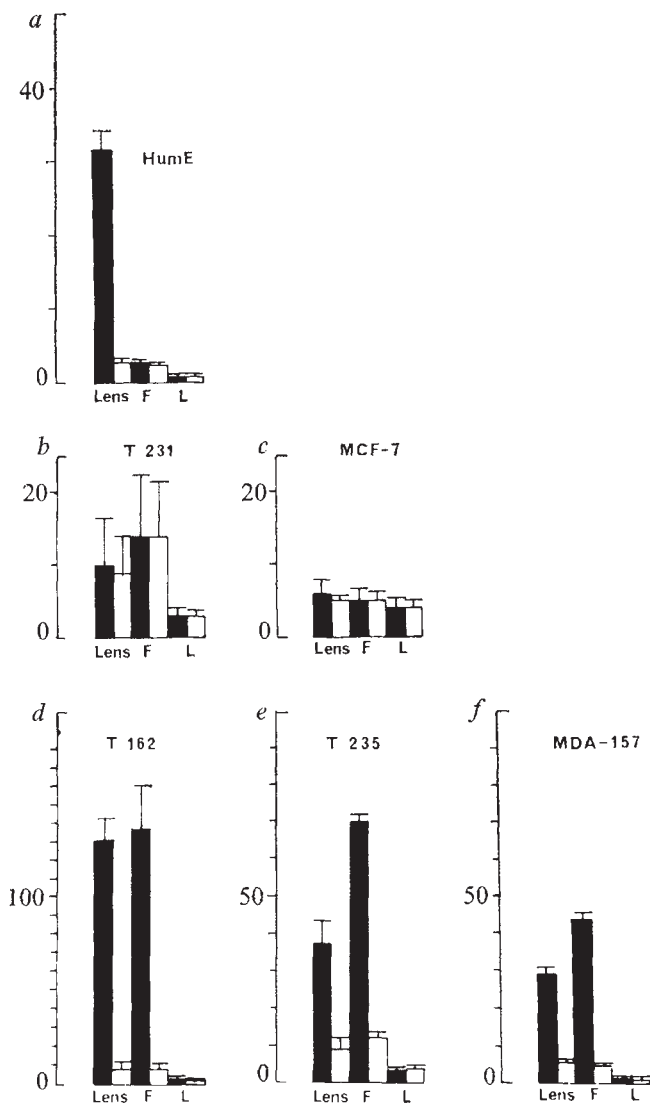


Fig. 1 Grain counts from autoradiographs, showing mean $\pm$ s.e. Closed blocks, recipients touching donors. Open blocks, non-touching recipients. Donor cells were seeded at approximately 6×10^3 cells per 35-mm dish and recipients at 2.5×10^5 per 50-mm dish in the relevant medium (L cells: Dulbecco's modified Eagle's medium with 10% foetal calf serum (FCS); MDA-157 and calf lens: medium 199 with 10% FCS; HumE (ref. 1) and HumF: medium 199 with 20% human serum, 15% FCS, insulin $10 \mu\text{g ml}^{-1}$, hydrocortisone $5 \mu\text{g ml}^{-1}$). After overnight incubation at 37°C , $2 \mu\text{Ci ml}^{-1}$ ³H-uridine was added to the donors for 3 h. The cells were then washed three times with medium, and recipient cells, which had just been detached by trypsinisation, were added in medium (199 with 20% human serum, 15% FCS, insulin and hydrocortisone). The donors and recipients were cocultured for 3 h at 37°C , fixed and processed for autoradiography⁸. In each experiment five donor dishes were used and tested with calf lens (twice), HumF (twice) and L cells (once). Experiments were repeated three times and on each dish, grain counts were made over ten recipients touching donors, and over ten recipients touching neither donors, nor other recipients. a, HumE; b, T231; c, MCF-7; d, T162; e, T235; f, MDA-157.

- ³ Azarnia, R., Larsen, W. J. & Loewenstein, W. R. *Proc. natn. Acad. Sci. U.S.A.* 71, 880 (1974).
⁴ Loewenstein, W. R. & Kanno, Y. *J. Cell Biol.* 22, 565 (1964).
⁵ Borek, C., Higashino, S. & Loewenstein, W. R. *J. membr. Biol.* 1, 274 (1969).
⁶ Pitts, J. D. & Simms, J. W. *Expl Cell Res.* 104, 153 (1977).
⁷ Subak-Sharpe, J., Burk, R. R. & Pitts, J. D. *J. cell. Sci.* 4, 353 (1969).
⁸ Fentiman, I. S., Taylor-Papadimitriou, J. & Stoker, M. *Nature* 264, 760 (1976).
⁹ Pitts, J. D. & Burk, R. R. *Nature* 264, 762 (1976).
¹⁰ Lasfargues, E. Y. & Ozzello, L. *J. natn. Cancer Inst.* 21, 1131 (1958).
¹¹ Hallowes, R. C., Millis, R., Stoker, M. & Taylor-Papadimitriou, J. *clin. Oncol.* 3, 81 (1977).
¹² Rose, H. N. & McGrath, C. M. *Science* 190, 673 (1975).
¹³ Young, R. K., Cailleau, R. M., Mackay, B. & Reeves, W. J. *In vitro* 9, 239 (1974).
¹⁴ Sheridan, J. D. *J. Cell Biol.* 45, 91 (1970).

Growing central axons deprived of normal target neurones by neonatal X-ray irradiation still terminate in a precisely laminated fashion

THE development of the central nervous system is characterised by the formation of specific synaptic connections. Highly selective connections form not only between classes of neurones, but also between specific neurones within classes. An additional degree of specificity is observed when the presynaptic axons contact only a limited portion of the target neurone. This kind of specificity is conspicuous in the dentate gyrus (fascia dentata) of the mammalian cerebral cortex. We describe here a situation in which the dentate gyrus offers a unique opportunity to test some of the hypotheses that may explain the selective termination of different afferents on restricted portions of the surface of a neurone.

The most numerous neurones in the dentate gyrus are the granule cells, whose cell bodies are gathered in a dense layer from which the dendrites radiate towards the surface of the brain through the molecular layer (Fig. 1a). The predominant afferent fibre systems that converge on the granule cells are spatially segregated, with little or no overlap, in three distinct zones across the dendritic trees. The outer zone receives fibres from the lateral entorhinal cortex of the temporal lobe, the middle zone is innervated by the medial entorhinal cortex, and the inner zone receives afferents from the hilus fasciae dentatae of both hemispheres¹⁻³ (Fig. 1a).

The majority of granule cells develop postnatally, whereas the sources of their major afferents are generated before birth⁴. Proliferating granule cell precursors are, unlike differentiating or mature neurones, extremely radiosensitive. This allows selective reduction of the granule cell population, while the afferent supplies would be expected to remain intact⁵. The granule cells arise in a temporal sequence from lateral to medial along the curvature of their layer⁴⁻⁶ and irradiation that reduces the number of cells in the lateral limb of the U-shaped granule cell layer totally eliminates the greater part of the medial limb⁵.

We have studied the fate of the axons that normally terminate in a highly selective laminar fashion in the molecular layer of the medial limb of the dentate gyrus when this target is removed by neonatal X-ray irradiation. Rat pups were shielded with lead, except for a field above the hippocampus and the dentate gyrus, and exposed to X rays from a 250-kV source at a distance of 34 cm. At a rate of 400 min⁻¹, 200 R was delivered on postnatal days 2, 3, 5 and 7. A comparable schedule has been shown to irreversibly reduce the number of granule cells to about 20%⁵. The animals were allowed to live for 2-4.5 months when selective lesions were made in the medial or lateral entorhinal cortex or the commissural and association pathways to the dentate gyrus. After a 3-d survival period the animals were killed and histological sections through the brains made and impregnated with silver to show degenerating axon terminals⁷ or treated according to a histochemical method for heavy metals⁸ which in normal brains precisely displays the terminal

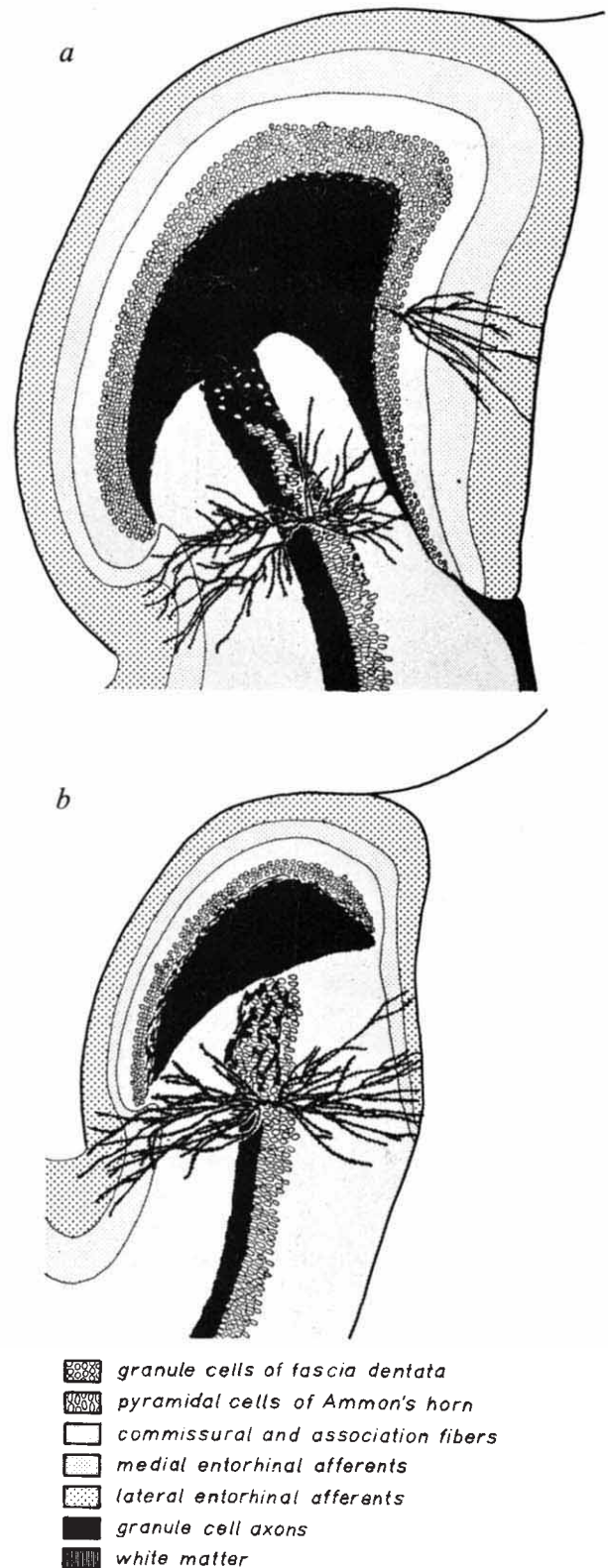


Fig. 1 Schematic drawings of sections through the dentate gyrus and adjacent part of Ammon's horn from *a*, a normal and *b*, a neonatally X-ray irradiated rat. The terminal zones of the converging fibre pathways are shown by different shadings. Three cells drawn from Golgi preparations are plotted on the diagrams. Directions: medial—right, anterior—bottom. $\times 50$.

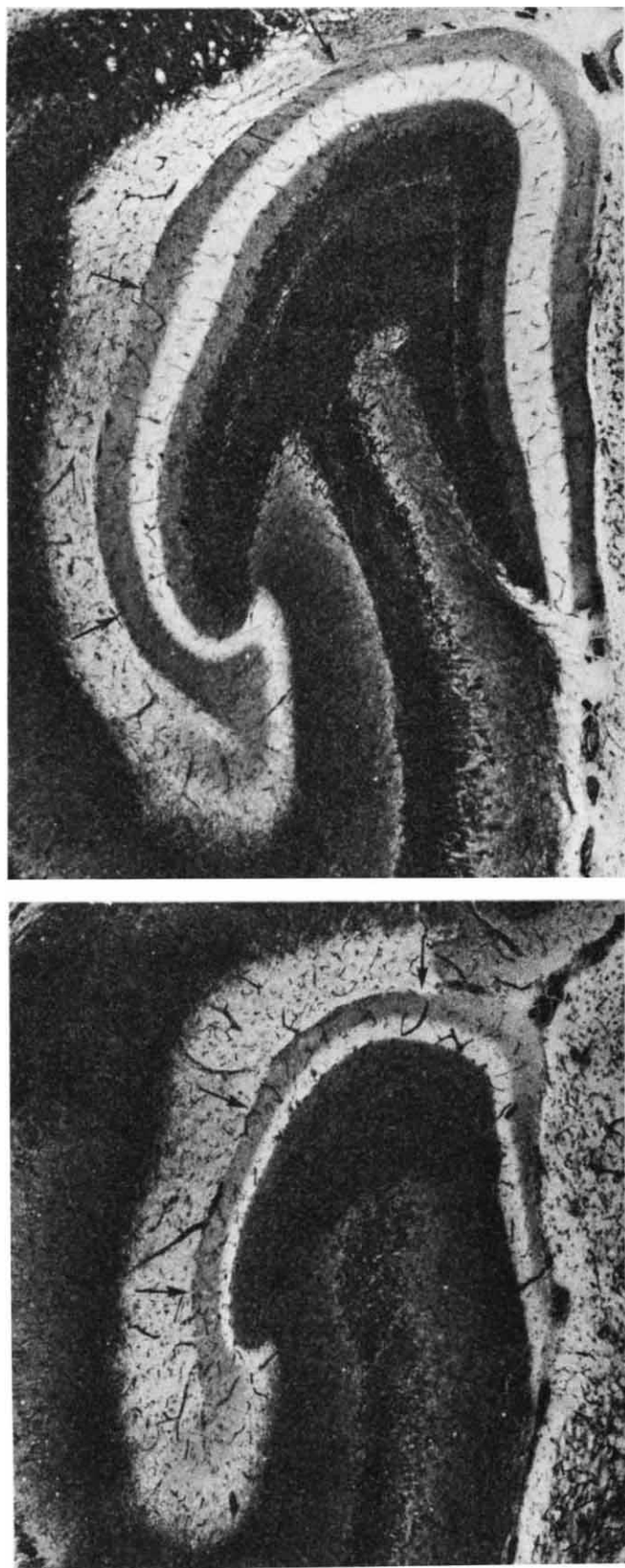


Fig. 2 Section through the dentate gyrus of a normal (top) and a neonatally X-ray irradiated (bottom) rat brain. The sections were stained with a histochemical method for heavy metals⁹ and with thionine, they display in different shades the terminal zones of the afferent pathways also shown in Fig. 1. Arrows point to the hippocampal fissure. $\times 45$.

zones of the three major systems of afferents to the dentate gyrus (Fig. 2).

In the irradiated animals, a histochemically distinct stratification is found not only in the molecular layer above the surviving granule cells but also medially where granule cells are absent (Figs 1, 2). The deep zone in this part is normally delimited by the granule cell layer; however, in the irradiated animals it merges with the stratum oriens beneath the pyramidal cell layer of the Ammon's horn. The degeneration material shows that each histochemically defined zone corresponds to an equally distinct terminal zone of one of the three major systems of afferents to the dentate gyrus. Degeneration in the inner zone is illustrated as an example (Fig. 3). Sections prepared according to the Golgi method, which impregnates single neurones, show that the medially located terminal zones of the entorhinal projections in the absence of granule cells are penetrated by basal dendrites of pyramidal cells of the adjacent Ammon's horn (Fig. 1b). These clearly elongated dendrites normally do not penetrate the terminal zones of the entorhinal fibres (Fig. 1a).



Fig. 3 Section through the dentate gyrus of a neonatally X-ray irradiated rat brain showing terminal degeneration of commissural and association fibres (compare Figs 1, 2). Fink-Heimer silver impregnation. Arrows point to the hippocampal fissure. $\times 45$.

These findings indicate that the laminar distribution of the terminal fields of the pathways converging on the dentate gyrus can exist without the developing axons selectively recognising particular coded portions of the normal target cell surface. This principle is similar to that based on observations made on the laminar distribution of afferents to the piriform cortex and to the dentate gyrus in the reeler mutant mouse. In both locations in the reeler, the termination is relatively independent of the location of the abnormally located target neurones^{9,10,17}.

It has been suggested that the complementary ordering of entorhinal afferents and commissural and ipsilateral association fibres to the molecular layer might be accounted for by a sequential arrival of the two groups of fibres¹¹. The earliest stage examined so far is that of the third postnatal day. At this time, the afferents from the medial and lateral entorhinal cortices and the ipsilateral association fibres have already arrived at the molecular layer and attained the same topographic relationship to each other as they do in mature brains. But, the

commissural projection to the deep zone arrives several days later and terminates in the same field as the ipsilateral association fibres. This illustrates that a sequential arrival of different classes of afferents does not necessarily result in a spatial segregation of their terminal ramification¹².

The abnormal growth of the basal dendrites of pyramidal cells close to the dentate gyrus is probably not a direct effect of radiation, since it has been shown that low-level X-ray irradiation of other parts of the cerebral cortex of immature rats results in dendrites that are shorter than normal¹³. The remarkable extension of the dendrites seen in this study is not understood. It may occur because the dentate gyrus normally hinders further growth and has been removed or, more likely, because the afferents deprived of normal recipients induce the abnormal growth. There are several examples suggesting that dendritic growth is influenced by afferent fibres¹⁴. The preparations described here suggest that neurones may adapt the size of their receptive surface in response to the amount of available afferents. While a reduction in dendritic size, on removal of afferents, has been described^{15,16}, this preparation indicates that the adaptation may also be in the nature of an increase in size.

This work was supported by the Danish MRC, the Danish Cancer Society and Den lægevidenskabelige Forskningsfond i Nordjyllands Amtskommune. We thank I. M. Jensen, K. Kirkholt, B. Krunderup, A. Meier, T. Nielsen and M. Sørensen for valuable technical assistance, and M. Hjelm-Hansen for assistance with the radiation procedure and dosimetry.

SØREN LAURBERG
ANDERS HJORTH-SIMONSEN

*Institute of Anatomy, University of Aarhus and
Institute of Cancer Research, Radiumstationen,
DK-8000 Aarhus C, Denmark*

Received 29 April; accepted 14 July 1977.

- ¹ Steward, O. J. *comp. Neurol.* **167**, 285–314 (1976).
- ² Hjorth-Simonsen, A. *J. comp. Neurol.* **146**, 219–232 (1972).
- ³ Hjorth-Simonsen, A. & Laurberg, S. *J. comp. Neurol.* **174**, 591–606 (1977).
- ⁴ Angevine, J. B., Jr *Exptl. Neurol. suppl.* **2**, 1–70 (1965).
- ⁵ Bayer, S. A. & Altman, J. *J. comp. Neurol.* **163**, 1–20 (1975).
- ⁶ Schlessinger, A. R., Cowan, W. M. & Gottlieb, D. I. *J. comp. Neurol.* **159**, 149–176 (1975).
- ⁷ Hjorth-Simonsen, A. *Stain Technol.* **45**, 199–204 (1970).
- ⁸ Haug, F.-M. S. *Adv. Anat. Embryol. Cell Biol.* **47**, 1–71 (1973).
- ⁹ Devor, M., Caviness, V. S., Jr & Derer, P. *J. comp. Neurol.* **164**, 471–482 (1975).
- ¹⁰ Stanfield, B. B., Caviness, V. S., Jr & Cowan, W. M. *Neurosci. Abst.* **1**, 1190 (1975).
- ¹¹ Loy, R., Lynch, G. & Cotman, C. W. *Brain Res.* **121**, 229–243 (1977).
- ¹² Fricke, R. & Cowan, W. M. *J. comp. Neurol.* **173**, 231–250 (1977).
- ¹³ Hicks, S. P. & D'Amato, J. in *Advances in Teratology* (ed. Woolam, D. H. M.) 195–250 (Logos, London, 1966).
- ¹⁴ Cotman, C. W. & Banker, G. A. in *Reviews of Neuroscience* (eds Ehrenpreis, S. & Kopin, I. J.) 1–62 (Raven, New York, 1974).
- ¹⁵ Bradley, P. & Berry, M. *Brain Res.* **109**, 133–151 (1976).
- ¹⁶ Jones, W. H. & Thomas, D. B. *J. Anat.* **96**, 375–381 (1962).
- ¹⁷ Stirling, V. R. & Bliss, T. V. P. *Brain Res.* (in the press).

Corollary discharge to cockroach giant interneurons

A PROBLEM faced by animals as they move about is to distinguish between sensory input which is generated as a direct consequence of the animals' own movements (called refference¹), and that generated by independent external events. One way an animal might handle refference would be to use an approximate copy of motor output (corollary discharge²) to repress or otherwise compensate for it. Cockroaches usually react to gentle puffs of air by rapidly running away, the so-called 'escape response'. Since they do not respond similarly to air currents set up by their own walking (even though the receptors involved are very sensitive to slight air movements from any direction³), they must have some mechanism for recognising and ignoring refferent input. The escape response had originally been thought to be initiated by the activity of the large interneurons known as

giant fibres⁴. This view has been disputed^{5,6}, however, and the function of the giants is at present unclear. I report here evidence that some of these giants are directly excited during walking by neural input which may represent corollary discharge from motor centres in the thorax, and which may aid the insect in handling refferent input.

Neural activity in the ventral nerve cord may be monitored during walking in a cockroach by suspending and eviscerating the insect, and recording with suction electrodes while the animal walks on a lightweight styrofoam ball, rotating this ball beneath itself⁷. In these conditions, several units become active (Fig. 1*a*). These units, identified as giant fibres on the basis of their large amplitudes and high conduction velocities^{4,7}, are considerably more active during walking (or in response to stimuli such as air puffs), than when the animal is quiet and inactive (Fig. 1*b*). That the activity of these neurones is not an artefact of the experimental arrangement is suggested by the finding that a similar pattern, that is, activity of giants during walking, near-silence during rest, may also be seen in animals which can move about freely (Fig. 1*c, d*).

The GFs receive input in the last abdominal ganglion from primary afferents arising at the bases of wind-sensitive hairs on the cerci, a pair of small, peg-like appendages at the end of the abdomen. Since I have observed that all action potentials in the giants during walking travel toward the head, the most obvious explanation for the observed activity is refference through the cerci; that is, excitation of the cercal afferents by air currents set up by the animal's own movements. Removal of the cerci seemed to support this idea, because it caused a marked reduction of neural activity

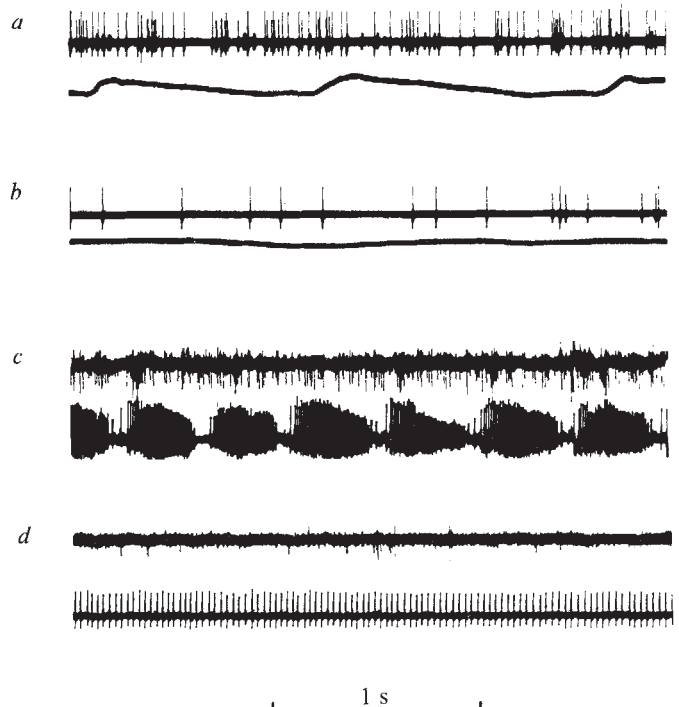


Fig. 1 Records of neural activity (top traces) in the ventral nerve cord of a single cockroach during walking (*a, c*) and at rest (*b, d*). *a, b*, Giant fibre activity in a suspended animal holding a styrofoam ball. The lower trace shows a monitor of the movement of one rear leg. Downward deflection represents extension. Spontaneous non-giant neural activity present in the nerve cord is hidden in the baseline and not visible in these records due to the low level of amplification used to record from the giant fibres. *c, d*, Activity in a free-walking animal. The lower trace shows the activity of the main femoral extensor muscle of one leg. Bursts of activity in this muscle indicate that the animal is walking (*c*). The muscle has a steady, low rate of firing when the cockroach is standing still (*d*). Note that very much higher levels of firing occur in the ventral cord during walking than at rest.

in the ventral nerve cord during walking. (It does not have any noticeable effect on the walking itself.) I have observed, however, that in intact animals the cercal nerves are never silent, even in a quiet, still room. It seems likely, therefore, that some of this continuous barrage of input from the cerci may provide subthreshold depolarisation to the recipient giant fibres such that any additional input would be enough to fire them. If the background input were removed, the level of depolarisation of the giants caused by other inputs might then be insufficient for the giant fibres to show much activation during walking.

I tested experimentally the hypothesis that there are non-cercal inputs driving the giants. First, I set up an eviscerated, suspended animal with a styrofoam ball as described above⁷. I then arranged a rubber hose over the abdomen of the cockroach so that a continuous stream of air could be blown posteriorly over the cerci. If the giant fibre activity which could be recorded during walking were only, or largely, the consequence of air currents set up by the animal's own movements, then recordings of ventral nerve cord activity during walking in the air stream should show no difference from similar recordings from quiet animals in the air stream. On the other hand, if refference from the cerci due to stimulation by air currents were not the main source of the activity, one would expect to see an increase in activity during walking even in the presence of the air stream. The latter turned out to be the case.

show no difference from similar recordings from quiet cockroach there is an initial burst of activity in the giant fibres in the ventral nerve cord which settles in to fairly high-frequency firing for a minute or more (Fig. 2*a*). If the velocity of the air stream is high enough this activity will soon die away (even though the air is still on) (Fig. 2*b*). In the stream of air, even vigorous puffs of air directed at the cerci from only a few centimetres away elicit no responses from the giant fibres (Fig. 2*c*), suggesting that in this situation the animal cannot generate air currents capable of eliciting any giant fibre response. Nevertheless, when the animal walks, one still observes the strong activity of the GFs also seen in animals not in an air stream (Fig. 2*d*). This activity disappears when the animal is again quiet (Fig. 2*e*).

While these results show that the giant fibre activity observed during walking is not brought about by stimulation of hair receptors by air currents, they do not eliminate the possibility that other receptors on the cerci or elsewhere could be stimulated by walking and thereby excite the giants. Cricket giant fibres, for example, are known to be very responsive to vibrational stimuli⁸. To test the possibility that refference from vibration-sensitive receptors in the posterior of the abdomen was responsible for giant fibre activity during walking, I arranged an animal so that the posterior third of its abdomen was supported by a small platform fastened to a wooden framework resting on the floor. The rest of the animal was supported at the thorax by a holder resting on the bench. By completely severing the abdomen, except for the nerve cord, between the platform and the other holder, it was possible to isolate mechanically the posterior part of the abdomen from the rest of the animal. Tests showed that vibrational stimuli which before the operation easily caused some of the giants to fire failed to do so after the operation. Nevertheless, giant fibre activity concomitant with walking was still apparent. To eliminate the effects of air currents in this situation, I also placed animals treated like this in an air stream, or coated their cerci with petroleum jelly. In no case was the giant fibre activity elicited by walking eliminated.

I conclude from these experiments that refference from cercal or other receptors during walking is not sufficient to account for the giant fibre activity which can be observed. Instead, it seems that the animal actively turns on some of the giants, by sending excitatory signals through small fibres down to the last abdominal ganglion from the thorax or

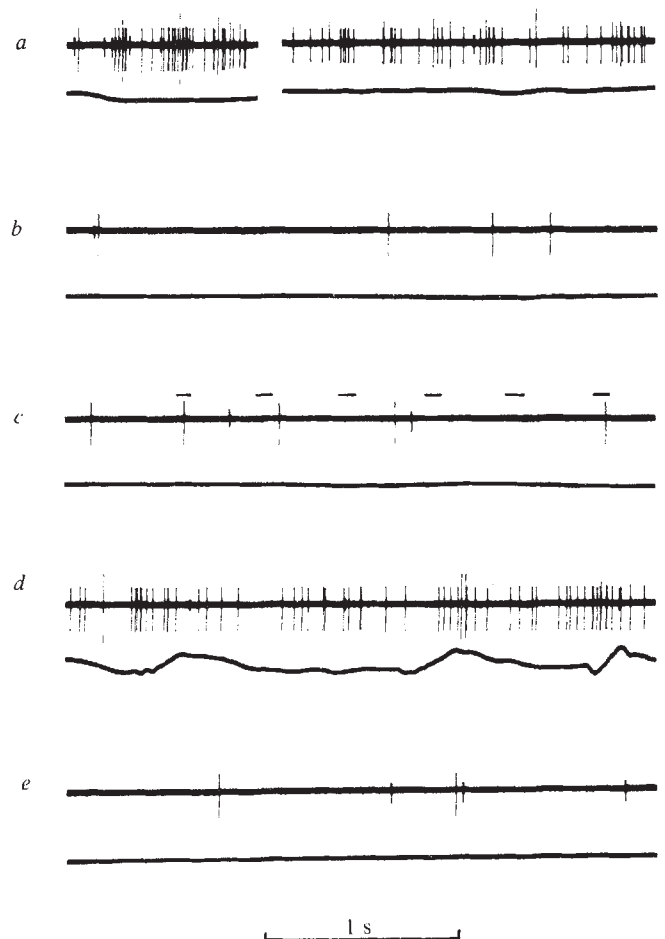


Fig 2. Records of neural activity in a suspended cockroach in the presence of a continuous, tailward stream of air. Upper traces, ventral nerve cord record; lower traces, leg movement monitor (down is extension). *a*, Onset of the air stream (left) and activity 1 min later (right). *b*, Activity after several minutes. *c*, Activity during delivery of puffs of air (bars above the record) at the cerci. The lack of response to the puffs indicates that in the presence of the air stream, additional air currents are ineffective in eliciting giant fibre activity. *d*, Activity during walking, in the air stream. *e*, Activity during rest, still in the presence of the air stream.

higher centres. It should be possible to test this hypothesis by cutting the ventral nerve cord in the abdomen. Receptors posterior to the lesion would continue to provide their stimulatory input during walking, but the descending excitation would be eliminated, thereby eliminating or drastically reducing giant fibre activity during walking.

This experiment was performed. A cockroach was prepared as before and recordings taken from two positions on the same side of the ventral nerve cord, one just anterior to the last abdominal ganglion, and one at about the middle of the abdomen. After recording the neural activity at both sites during walking (Fig. 3*a*), the entire nerve cord was severed between the two recording electrodes. The effect of this procedure was a dramatic reduction in giant fibre activity recorded at the posterior electrode during walking (Fig. 3*b*). Cutting the nerve cord did not seem to change the responsiveness of the giants to cercal stimulation since gentle puffs of air still elicited vigorous firing in the intact segments of the giants posterior to the cut. The result therefore clearly supports the hypothesis that descending excitation to the giants, which accompanies motor activity in the legs, is the main factor in bringing about the activity during walking.

Compensating for refference can be a special problem for an animal which has sensitive sensory systems which can

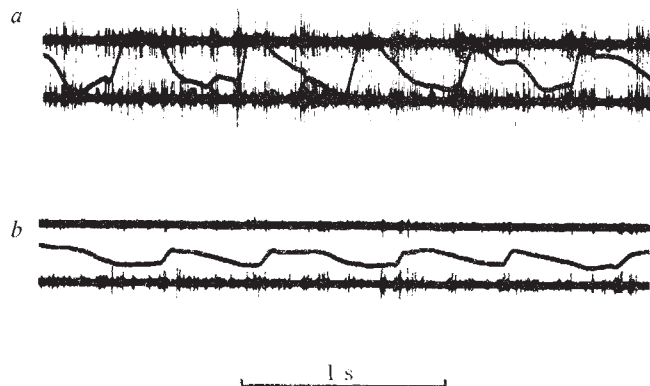


Fig. 3 Records of neural activity in a suspended cockroach before (a) and after (b) cutting the ventral nerve cord between the two recording electrodes. Top trace, recording from anterior-most electrode; bottom trace, recording from posterior-most electrode; middle trace, leg movement monitor (down is extension). Note the complete abolition of giant fibre activity in the anterior record (because the headward-moving spikes in the giant fibres cannot pass the cut) and the drastic reduction of activity in the posterior record after the ventral cord was cut.

be stimulated by the animal's own movements and whose activity can trigger evasive behaviour. Some fish and amphibians with lateral line systems handle the problem by suppressing the activity of the sensory systems, either centrally or peripherally^{9,10}. Insects may use this mechanism as well. The activity of some of the giant fibres can be inhibited during walking in the cricket¹¹ and in the cockroach (D. L. Daley, personal communication).

The results I report here show, however, that in cockroaches there is also a population of giants which is excited during walking. Since the excitation seems to derive from thoracic or higher centres and parallels movements of the legs, it can be considered to represent corollary discharge from the thoracic locomotor control centres. Excitation of some of the giant fibres by this corollary discharge presumably would have a role in allowing the animal to respond more quickly to escape-producing stimuli while it is walking, but at present even this possibility is only speculation, and the mechanism by which it would function is completely unknown.

D. L. Daley and B. J. Mendius made useful comments on the manuscript. The work was supported by a Bio-Medical Science Support Grant and USNIH grant NS12142.

FRED DELCOMYN

Department of Entomology and
Program in Neural and Behavioral Biology,
University of Illinois,
Urbana, Illinois 61801

Received 9 May; accepted 11 July 1977.

- ¹ von Holst, E. & Mittlestaedt, H. *Naturwissenschaften* 37, 464–476 (1950).
- ² Sperry, R. W. J. *comp. physiol. Psychol.* 43, 482–489 (1950).
- ³ Nicklaus, R. Z. *vergl. Physiol.* 50, 331–362 (1965).
- ⁴ Roeder, K. D. J. *exp. Zool.* 108, 243–261 (1948).
- ⁵ Dagan, D. & Parnas, I. J. *exp. Biol.* 52, 313–324 (1970).
- ⁶ Harris, C. L. *Comp. Biochem. Physiol.* 56A, 333–335 (1977).
- ⁷ Delcomyn, F. J. *Insect Physiol.* 22, 1223–1227 (1976).
- ⁸ Edwards, J. S. & Palka, J. *Proc. R. Soc. B* 185, 83–103 (1974).
- ⁹ Russell, I. J. *J. exp. Biol.* 54, 621–641 (1971).
- ¹⁰ Russell, I. J. *J. comp. Physiol.* 111, 334–358 (1976).
- ¹¹ Murphey, R. K. & Palka, J. *Nature* 248, 249–251 (1974).

Regulation of adenylate cyclase activity in glial–adrenal hybrid cells

SOMATIC cell hybrids, formed by fusion of hormone-responsive and insensitive cells, have been used to evaluate the structure and genetic regulation of the hormone-sensitive adenylate cyclase system^{1–6}. In the cell hybrids studied so far, catecholamine-sensitive adenylate cyclase activity was lost when catecholamine-responsive and insensitive cells were fused. Prostaglandin E₁

(PGE₁)-sensitive adenylate cyclase activity, on the other hand, was retained in hybrids between PGE₁-sensitive and insensitive cells. Where examined, the presence or the absence of hormone sensitivity in the hybrids closely correlated with hormone receptor activity as measured by ligand-binding assays^{1–3}. These observations suggest that genetic mechanisms may control the expression of hormone responsiveness at the level of the hormone receptor. To examine these patterns of regulation further, we have formed cell hybrids between rat glial tumour cells with adenylate cyclase activity responsive to β -adrenoceptor agonists⁷ and mouse adrenocortical tumour cells with adrenocorticotrophic hormone (ACTH)-sensitive adenylate cyclase activity⁸. Besides having distinct functional hormone receptors, the adenylate cyclases of parental cells exhibit marked quantitative differences in response to fluoride ion^{7,8}. We find that the glial–adrenal hybrids retain the adrenaline-sensitive adenylate cyclase activity characteristic of the glial parent, and lose ACTH sensitivity. The level of the fluoride response, however, is characteristic of the adrenal parent. These data indicate that hormone receptor and catalytic unit activities in the adenylate cyclase system are independently regulated.

The clones used in the cell hybridisation experiments presented here are listed and described in Table 1. C6 cells were from a rat glial tumour cell line¹⁰; Y1 and OS3 adrenocortical cells were from mouse adrenocortical tumour cell lines^{11,12}. The isolation and characterisation of C6 cells deficient in thymidine kinase activity (TK[−]) has been described previously¹³. The isolation of Y1, OS3 and C6 clones deficient in hypoxanthine–guanosine phosphoribosyl transferase activity (HGPRT[−]) was accomplished by mutagenesis with ethyl methanesulphonate 300 μ g ml^{−1} and selection with 6-thioguanine 5 μ g ml^{−1}. The HGPRT[−] parents had less than 5% of the HGPRT activity¹⁴ of controls and did not form colonies when 10⁷ cells were plated in the presence of amethopterin¹⁵. Cell hybrids were prepared by fusing 10⁶ HGPRT[−] adrenal cells in monolayers with 10⁴ TK[−] glial cells, using chemically inactivated Sendai virus (Connaught Laboratories) as described by Watkins¹⁶. After fusion, hybrids were selectively grown in medium containing hypoxanthine, thymidine and amethopterin^{13,17}. The hybrid nature of the cells isolated in selective growth conditions was confirmed by analysis of karyotypes¹⁸. Cells isolated after fusing Y1_{HGPRT[−]} or OS3_{HGPRT[−]} cells with C6_{TK[−]} cells were polyploid with slightly fewer than 14 metacentric chromosomes denoting the contribution from one glial and one adrenal parent (Table 1).

The adenylate cyclase systems from glial and adrenal parental cells were readily distinguished by their responses to hormones and to fluoride. In the C6 cell lines, adenylate cyclase activity was increased by adrenaline, whereas in the Y1 cell line, the enzyme was stimulated by ACTH (Table 1). High levels of fluoride-stimulated enzyme activity (approximately 200 pmol cyclic AMP formed per min per mg protein) seemed to be a characteristic feature of the mouse adrenocortical tumour cells^{8,9,19}, and also was observed in the OS3 adrenal line, an ACTH-insensitive, Y1 cell variant (for example, Table 1). These levels of fluoride-stimulated adenylate cyclase activity were $\geq$ threefold those seen in C6 cells (ref. 7), in TK[−] and HGPRT[−] subclones (Table 1), or in C6_{TK[−]} $\times$ C6_{HGPRT[−]} hybrids (Table 1).

Hormone sensitivity in C6_{TK[−]} $\times$ Y1_{HGPRT[−]} hybrids resembled that of the C6_{TK[−]} glial cell parent. Adenylate cyclase activity was stimulated by adrenaline, but not by ACTH (Table 1). In the presence of fluoride, adenylate cyclase activity was $\geq$ 200 pmol cyclic AMP formed per min per mg protein, characteristic of the Y1_{HGPRT[−]} adrenal parent (Table 1). These patterns of expression were observed consistently in 11 different C6_{TK[−]} $\times$ Y1_{HGPRT[−]} hybrid populations, in four C6_{TK[−]} $\times$ OS3_{HGPRT[−]} hybrids, and also in clonal isolates from each (Table 1).

The retention of adrenaline sensitivity (β^+) and loss of ACTH sensitivity (ACTH[−]) in the adenylate cyclase system of glial (β^+ , ACTH[−])–adrenal (ACTH^{+, β^+) hybrids, suggest the presence of regulatory influences distinct from those reported previously^{1–4}. The high level of fluoride-stimulated adenylate cyclase activity observed in the glial–adrenal hybrids but not in glial–glial hybrids (Table 1) indicate the contributions of adrenal components}

Table 1 Properties of parental clones and hybrids

	Cell line	Chromosome number		Adenylate cyclase activity			NaF	n
		Total	Metacentric	Basal	Adrenaline	ACTH		
Parents	C6 _{TK} ⁻	35	14	7 ± 2	94 ± 1	ND	66 ± 6	4
	C6 _{HGPRT} ⁻	42	14	10 ± 1	81 ± 5	ND	61 ± 7	4
	Y1 _{HGPRT} ⁻	40	0	6 ± 1	ND	40 ± 3	217 ± 23	5
	OS3 _{HGPRT} ⁻	42	0	2	2	2	190	1
Hybrids	C6 _{TK} ⁻ × Y1 _{HGPRT} ⁻	78 (72-89)	12	13 ± 3	66 ± 16	14 ± 3	263 ± 31	11
	C6 _{TK} ⁻ cloned	83	ND	26	199	24	424	1
	C6 _{TK} ⁻ × OS3 _{HGPRT} ⁻	82 (73-88)	11	5 ± 3	60 ± 8	4 ± 2	231 ± 55	4
	C6 _{TK} ⁻ cloned	88 (79-95)	9	6	79	6	437	1
	C6 _{TK} ⁻ × C6 _{HGPRT} ⁻	72	ND	6 ± 1	43 ± 8	6 ± 1	59 ± 7	5

Chromosome numbers for parental lines are modal numbers based on scores from at least 50 spreads. Values for hybrids are either modal numbers or averages with the range given in parenthesis.

Adenylate cyclase activity is given as pmol cyclic AMP formed per min per mg protein ± s.e.m. The number of determinations is given under *n*. Adrenaline 33×10^{-5} M; ACTH_{1-24}} 2×10^{-5} M; and NaF 15×10^{-3} M were added where indicated. C6 cells are insensitive to ACTH and Y1 cells are insensitive to adrenaline^{7,9}. Activity was assayed in cell homogenates by conversion of labelled ATP to labelled cyclic AMP^{7,8}.

ND, Not determined.

The total hybrid population surviving each selection was analysed. Data were accumulated from the *n* hybrid preparations indicated in the last column.

determining the catalytic activity of the adenylate cyclase system. Glial components of the fluoride response may be present but masked. Membrane fusion experiments of Orly and Schramm²⁰ suggest that a receptor from one cell should be able to couple functionally with the adenylate cyclase from another cell. These observations raise the interesting possibility that the adenylate cyclase system in the glial-adrenal hybrids is a mixture of components from each parent.

We thank Drs J. Logothetopoulos and C. J. Ingles for discussions, Mrs Deborah Brymer for assistance and Dr W. Rittel (Ciba-Geigy) and Dr H. Strade (Organon) for gifts of ACTH_{1-24}}. This work was supported by the MRC of Canada.

BERNARD P. SCHIMMER
JENNIVINE TSAO
NGAI HOI CHEUNG

Banting and Best Department of
Medical Research,
University of Toronto,
112 College Street,
Toronto, Ontario M5G 1L6, Canada

Received 23 May; accepted 27 June 1977.

- Gilman, A. G. & Minna, J. D. *J. biol. Chem.* **248**, 6610-6617 (1973).
- Minna, J. D. & Gilman, A. G. *J. biol. Chem.* **248**, 6618-6625 (1973).
- Brunton, L. L., Maguire, M. E., Anderson, H. J. & Gilman, A. G. *J. biol. Chem.* **252**, 1293-1302 (1977).
- Hamprecht, B. & Schultz, J. *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 1633-1641 (1973).
- Ayad, S. R. & Foster, S. J. *Cell* **3**, 135-140 (1974).
- Sharma, S. K., Nirenberg, M. & Klec, W. A. *Proc. natn. Acad. Sci. U.S.A.* **72**, 590-594 (1975).
- Schimmer, B. P. *Biochim. biophys. Acta* **252**, 567-573 (1971).
- Schimmer, B. P. *J. biol. Chem.* **247**, 3134-3138 (1972).
- Taunton, O. D., Roth, J. & Pastan, I. *J. biol. Chem.* **244**, 247-253 (1969).
- Benda, P., Lightbody, J., Sato, G., Levine, L. & Sweet, W. *Science* **161**, 370-371 (1968).
- Yasumura, Y., Buonassisi, V. & Sato, G. H. *Cancer Res.* **26**, 529-535 (1966).
- Schimmer, B. P. *J. Cell Physiol.* **74**, 115-122 (1969).
- Schimmer, B. P., Stevenson, L. F., ter Hofstede, C., Cheung, N. H. & Marks, A. *Expl. Cell Res.* **86**, 425-428 (1974).
- Harris, H. & Cook, P. R. *J. Cell Sci.* **5**, 121-134 (1969).
- Littlefield, J. W. *Proc. natn. Acad. Sci. U.S.A.* **50**, 568-576 (1963).
- Watkins, J. in *Methods in Virology* 5 (eds Maramorosch, K. & Koprowski, H.) 1-32 (Academic, New York, 1971).
- Littlefield, J. W. *Science* **145**, 709-710 (1964).
- Rothfels, K. H. & Siminovich, L. *Stain Tech.* **33**, 73-77 (1958).
- Schimmer, B. P. *Nature* **259**, 482-483 (1976).
- Orly, J. & Schramm, M. *Proc. natn. Acad. Sci. U.S.A.* **73**, 4410-4414 (1976).

tricyclic antidepressant drugs may also have this activity. To test this hypothesis, four tricyclic antidepressants—representing four different structural types—were tested on the H₂ receptor linked to adenylate cyclase in homogenates of the guinea pig hippocampus and cortex. Both dimaprit and histamine were used as agonists. Dimaprit has potent activity at the H₂ receptor with negligible activity at the H₁ receptor, that is, less than 0.0001% of that of histamine².

Each experiment was done on fresh preparations of hippocampus and cortex from adult guinea pigs. The tissues were homogenised in a Potter-Elvehjem glass-Teflon vessel in 50 volumes of a medium containing 0.32 M sucrose, 5 mM Tris-chloride, 1 mM EGTA, and 1 mM dithiothreitol, pH 7.4. The method for measuring adenylate cyclase activity has been described⁸. The assay system contained 75 mM Tris-chloride (pH 7.4), 1 mM ATP, 2 mM MgCl₂, 1 mM cyclic AMP, 5 mM phosphocreatine, 0.5 mg ml⁻¹ creatine kinase (ATP creatine phosphotransferase, EC 2.7.3.2) 4 mM theophylline, 10⁻⁵ M GTP, 0.1 M sucrose, 0.3 mM EGTA, 0.3 mM dithiothreitol, and enzyme protein (150-190 µg) in a final volume of 225 µl. All assays were performed in triplicate. All additions were made to the assay tubes on ice. They were then transferred to a 30 °C shaking incubator and preincubated for 5 min to allow the enzymatic activity to reach a steady rate and to eliminate the influence of any lag periods in hormone activation. After the preincubation period, 25 µl α-³²P-ATP (1-2 Ci) were added and the reaction was allowed to proceed for 13 min when it was stopped by adding 100 µl of 1% sodium dodecylsulphate. After addition of 650 µl of ³H-cyclic AMP (5,000-10,000 c.m.p.) to monitor recovery, the labelled cyclic AMP was isolated with alumina and Dowex AG-50W (ref. 4). The reaction was linear with protein concentration⁵ in the range used and for at least 15 min after the addition of the α-³²P-ATP. Curve fitting techniques⁸ with the PROPHET (ref. 7) computer system were used to estimate the apparent ED₅₀ (50% effective dose) values, maximum stimulation by agonists, and parallelism of the dose-response curves. Antagonism was analysed by means of Schild plots^{8,9}.

All four tricyclic antidepressants shifted the dose-response curve of histamine- and dimaprit-stimulated adenylate cyclase in a parallel manner. Figure 1 shows histamine-stimulated adenylate cyclase activity in the absence and presence of amitriptyline. The competitive nature of antagonism by amitriptyline and imipramine was further revealed by Schild plots. As shown for amitriptyline (Fig. 2) the slope of the Schild plot, with either histamine or dimaprit as agonist, is 1.02, which is not significantly

Tricyclic antidepressant drugs block histamine H₂ receptor in brain

THE observation that cyproheptadine is a competitive antagonist of the histamine H₂ receptor linked to adenylate cyclase in brain¹ suggested that the chemically similar

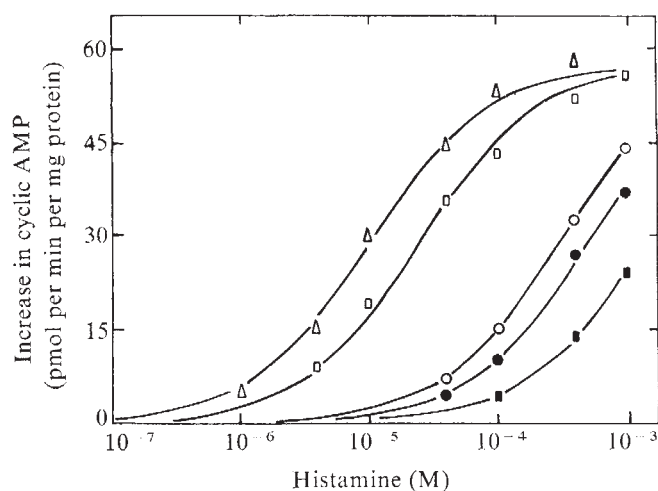


Fig. 1 Increase in adenylate cyclase activity of the guinea pig hippocampus in response to varying concentrations of histamine in the absence and presence of different concentrations of amitriptyline. Each point is the mean of triplicate determinations on a single enzyme preparation. Amitriptyline alone did not affect basal activity. Control, Δ ; amitriptyline at 4×10^{-7} M, $\square$; 10^{-6} M, $\circ$; 4×10^{-6} M, $\bullet$; and 10^{-5} M, $\blacksquare$.

different from unity. Table 1 summarises the pA_2 values of the four antidepressants. Amitriptyline is the most active drug, about as potent as cyproheptadine ($pA_2=7.43$, ref. 1). Dibenzepin and iprindole have about 100-fold less affinity for the cyclase. As the histamine-sensitive adenylate cyclase in broken cell preparations of guinea pig cortex and hippocampus respond identically to histamine, dimaprit and antagonists¹, it was appropriate to compare the sensitivities

Table 1 pA_2 values of tricyclic antidepressant drugs in inhibiting histamine- and dimaprit-stimulated adenylate cyclase activity in homogenates of guinea pig hippocampus

Drug	Histamine pA_2 values	Dimaprit
Amitriptyline	7.23 ± 0.27 (7)	7.47 ± 0.55 (7)
Imipramine	6.39 ± 0.16 (7)	6.61 ± 0.19 (6)
Dibenzepin	5.49 ± 0.11 (3)	5.81 ± 0.11 (3)
Iprindole	5.63 ± 0.11 (3)	5.49 ± 0.27 (3)

Values are means $\pm$ s.d. The numbers of different hippocampi that the drugs were tested on are given in parentheses.

of the cyclases in the cortex and hippocampus to tricyclic antidepressants. Figure 2 shows that the values obtained on homogenates from cortex are described by the same Schild plot as derived from observations on homogenates of the hippocampus.

It should be noted that these drugs also blocked dimaprit-activated adenylate cyclase activity in a competitive manner. Since dimaprit has negligible H_1 -antagonist activity² the inhibition of dimaprit-sensitive adenylate cyclase by tricyclic antidepressants permits their classification as histamine H_2 antagonists. The pA_2 values of H_2 antagonists on histamine- or dimaprit-activated adenylate cyclase activity in homogenates of guinea pig hippocampus^{3,10}, and cortex¹, and cardiac ventricles³ were very nearly identical to the pA_2 values on pharmacological preparations thus showing that blockade of this adenylate cyclase in these tissues reflect pharmacological effects on the H_2 receptor in other tissues.

Brain concentration of imipramine after a single dose of 15 mg per kg body weight intramuscularly was found¹¹ in

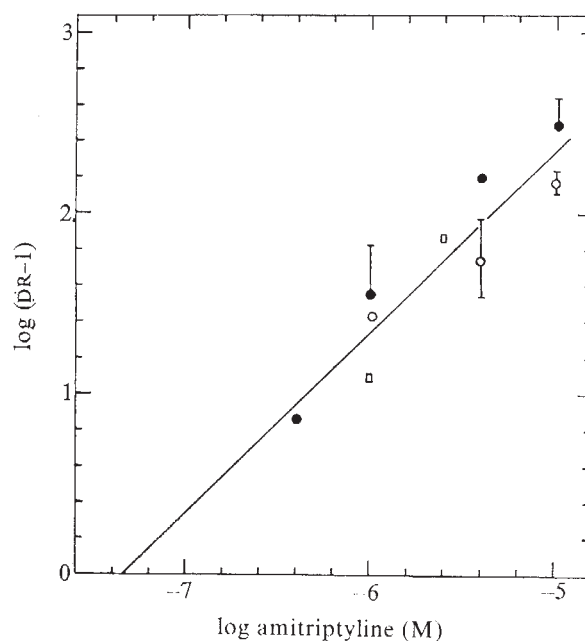


Fig. 2 Schild plot for inhibition by amitriptyline of dimaprit- and histamine-stimulated adenylate cyclase activity in the guinea pig hippocampus and cortex. The dose ratios (DR) were calculated as the ratio of the ED_{50} of histamine in the presence and absence of different concentrations of amitriptyline (B). Simple competitive antagonism results in a straight line of slope 1 when $\log(DR-1)$ is plotted against $\log B$; and the intercept with the abscissa is $-\log K_B$, where K_B is the apparent dissociation constant for the antagonist-receptor interaction; $-\log K_B$ is referred to as pA_2 . The slope of the line is 1.02, not significantly different from unity, and the pA_2 value is 7.35. Each point is the mean of experiments on tissue from at least two animals. Dimaprit stimulated activity in $\bullet$, hippocampus and $\square$, cortex. $\circ$, Histamine stimulated activity in hippocampus.

Table 2 Potencies of some tricyclic antidepressant drugs in blockade of the histamine H_2 receptor, the muscarinic receptor, and in inhibiting uptake of 5-hydroxytryptamine (5-HT) and noradrenaline (NA)

Drug	Anti- H_2 activity		Antimuscarinic activity		IC ₅₀ (M) for inhibition of uptake:	
	K_B^* (M)	IC ₅₀ [†] (M)	K_B^* (M)	K_B^* (M)	5-HT (ref. 14)	NA (ref. 15)
Amitriptyline	3.4×10^{-8}	6.8×10^{-8}	3.3×10^{-8}	8.3×10^{-9}	4.9×10^{-7}	5.5×10^{-8}
Imipramine	2.4×10^{-7}	4.8×10^{-7}	2.0×10^{-7}	6.5×10^{-8}	2.8×10^{-7}	1.0×10^{-6}
Dibenzepin	1.5×10^{-6}	3.0×10^{-6}	1.8×10^{-6}	—	—	—
Iprindole	3.2×10^{-6}	6.4×10^{-6}	—	—	1.0×10^{-5}	—

*Dimaprit as agonist, from Table 1.

[†]Calculated according to: $IC_{50} = K_B(1 + [A]/K_A)$, where K_B = the apparent dissociation constant for the drug-receptor interaction; $[A]$, agonist concentration, taken arbitrarily as $[A] = 2 \times K_A$; and K_A is the apparent dissociation constant for the dimaprit-receptor interaction which equals the ED_{50} value.

[‡]From ref. 12, where 3H -N-methyl-4-piperidylbenzilate was used as the labelled ligand ($K_B = 5 \times 10^{-10}$ M), at a concentration of 5×10^{-10} M.

[§]Calculated from ref. 13, where 3H -3-quinuclidinylbenzilate was used as the labelled ligand ($K_B = 3 \times 10^{-10}$ M), at a concentration of 6×10^{-11} M.

rat to be $1,500\mu\text{g l}^{-1}$, which corresponds to $5.4 \times 10^{-6}\text{ M}$. This concentration is higher than the apparent K_B value (Table 2), suggesting that the brain H_2 receptors could be occupied *in vivo* after administration of pharmacologically active doses. Among the mechanisms proposed to account for the pharmacological effects of the tricyclic antidepressants are blockade of re-uptake of noradrenaline and of 5-hydroxytryptamine and blockade of muscarinic receptors. Table 2 shows the potency of these drugs at these sites and at the H_2 receptor. Their K_B values at the H_2 receptor and muscarinic receptor (column 3 of Table 2) are surprisingly similar. The IC_{50} values (concentrations for 50% inhibition) for uptake cannot be directly compared with the IC_{50} values for blocking the H_2 receptor because the affinities of 5-hydroxytryptamine and noradrenaline for the uptake system are not known; but the estimates (Table 2) suggest that potency for H_2 blockade is at least comparable with potency in blocking uptake of the amines.

Nevertheless, the information available cannot support an assertion that blockade of the H_2 receptor (or any other single action of these drugs) contributes to their therapeutic effect. But some of the pharmacological effects of the antidepressant drugs may rest on blockade of H_2 receptors. For example, histamine increases fluid consumption in rats by a central mechanism¹⁶⁻¹⁸, and tricyclic antidepressant drugs diminish fluid consumption in rats¹⁹. One of the rare adverse effects of these drugs is hallucinations²⁰; perhaps pertinent is the finding that the hallucinogen, *d*-lysergic acid diethylamide, also blocks the H_2 receptor¹. Of special and practical interest is that amitriptyline has greater affinity for the H_2 receptor (Table 1) than does cimetidine ($pA_2=6.22$; ref. 1), the H_2 antagonist that is so effective in treating peptic ulcer^{21,22}. The antimuscarinic activity of some of the tricyclic antidepressants should further enhance their usefulness in treating this disease. Like cimetidine, antidepressant drugs have been shown to decrease gastric acidity in man²³ and gastric acid secretion in laboratory animals²⁴⁻²⁷ and to prevent gastric ulcer formation in animals^{24,27-30}—effects that were also noted with an antidepressant drug lacking antimuscarinic activity²⁷.

This research was supported by a grant from the US National Institute of Mental Health.

J. P. GREEN
S. MAAYANI

Department of Pharmacology,
Mount Sinai School of Medicine of
the City University of New York,
New York, New York 10029

Received 20 June; accepted 11 July 1977.

- ¹ Green, J. P., Johnson, C. L. & Weinstein, H. in *Histamine Receptors* (ed. Yellin, T. O.) (Spectrum, New York, in the press).
- ² Parsons, M. E., Owen, D. A. A., Ganellin, C. R. & Durant, G. J. *Ag. Actions* 7, 31-38 (1977).
- ³ Johnson, C. L. & Mizoguchi, H. *J. Pharmac. exp. Ther.* 200, 174-186 (1977).
- ⁴ Salomon, Y., Londres, C. & Rodbell, M. *Analyt. Biochem.* 58, 541-548 (1974).
- ⁵ Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* 193, 265-275 (1951).
- ⁶ Waud, D. R. & Parker, R. B. *J. Pharmac. exp. Ther.* 177, 13-24 (1971).
- ⁷ Raub, W. F. *Fedn Proc.* 33, 2390-2392 (1974).
- ⁸ Schild, H. O. *Pharmac. Rev.* 9, 242-246 (1957).
- ⁹ Arunlakshana, O. & Schild, H. O. *Br. J. Pharmac.* 14, 48-58 (1959).
- ¹⁰ Hegstrand, L. R., Kanof, P. D. & Greengard, P. *Nature* 260, 163-165 (1976).
- ¹¹ Nagy, A. *J. Pharm. Pharmac.* 29, 104-107 (1977).
- ¹² Rehavi, M., Maayani, S. & Sokolovsky, M. *Biochem. Pharmac.* (in the press).
- ¹³ Snyder, S. H. & Yamamura, H. I. *Archs gen. Psych.* 34, 236-239 (1977).
- ¹⁴ Horn, A. S. & Trace, S. H. *Br. J. Pharmac.* 51, 399-403 (1974).
- ¹⁵ Horn, A. S., Coyle, J. T. & Snyder, S. H. *Molec. Pharmac.* 7, 66-80 (1971).
- ¹⁶ Gerald, M. C. & Maickel, R. P. *J. Pharmac.* 44, 462-471 (1972).
- ¹⁷ Gutman, Y. & Krausz, M. *Eur. J. Pharmac.* 23, 256-263 (1973).
- ¹⁸ Liebowitz, S. F. *Brain Res.* 63, 440-444 (1973).
- ¹⁹ Zabik, J. E., Levine, R. M., Spaulding, J. H. & Maickel, R. P. *Neuropharmacology* 16, 267-271 (1971).
- ²⁰ Klerman, G. L. & Cole, J. O. *Pharmac. Rev.* 17, 101-141 (1965).
- ²¹ Editorial *Br. med. J.* 1, 1275-1278 (1976).
- ²² Editorial *J. Am. med. Ass.* 237, 2224 (1977).
- ²³ Varay, A., Berthelot, J., Billiotet, J., Veterbo, G. & Graf, B. *Bull. Mém. Soc. méd. Hôp. Paris* 76, 228-239 (1960).
- ²⁴ Bonfils, S., Dubrasquet, M. & Lambling, A. *J. appl. Physiol.* 17, 299-300 (1962).
- ²⁵ Bass, P. & Patterson, M. A. *J. Pharmac. exp. Ther.* 156, 142-149 (1967).
- ²⁶ Lippmann, W. *Biochem. Pharmac.* 18, 2517-2529 (1969).
- ²⁷ Lippmann, W. & Seethaler, K. *Life Sci.* 20, 1393-1400 (1977).
- ²⁸ Garattini, S., Giachetti, A., Jori, A., Pieri, L. & Valzelli, L. *J. Pharm. Pharmac.* 14, 509-514 (1962).
- ²⁹ Lippmann, W. *J. Pharm. Pharmac.* 22, 568-573 (1970).
- ³⁰ Lippmann, W. *Archs Int. Pharmacodyn.* 193, 340-356 (1971).

Reversal of the Na-K pump and apparent affinity for intracellular potassium

CENTRAL to all formulations of the transport mechanism of the membrane Na-K pump should be the description of cation binding and release. The acceptance sites for transport by the pump, external sites for K and internal sites for Na, have been extensively studied, and linked to the activating sites of the associated Na,K-ATPase¹. By contrast, little is known about the discharge sites of the pump, although proposed characteristics of these sites have vital roles in various transport and enzyme schemes, such as cyclical conversions of discharge sites for one cation into acceptance sites for the other. We have studied the apparent affinity for K at its intracellular discharge sites by measuring the rate of ATP synthesis as a function of the internal K concentration in resealed red blood cell ghosts, where the Na-K pump is driven in reverse by the downhill efflux of K and influx of Na: the observed $K_{0.5}$ for internal K was 0.3 M.

Resealed ghosts were prepared from fresh, human red blood cells, following the procedure of Bodemann and Passow² and Bodemann and Hoffman³. Cells, washed three times in 160 mM choline chloride-10 mM Tris-HCl (pH 7.4), were suspended at 50% haematocrit in isotonic Tris-HCl (166 mM, pH 7.4) at 0 °C, and then rapidly mixed with 10 volumes of ice-cold haemolysing solution⁴ containing 1 mM ATP, 2 mM ADP, 9 mM MgCl₂, 10 mM iodoacetate, and 5 mM ³²P-phosphate. After 5 min the salt concentration was restored to 160 mM by adding 3.2 M KCl (or KCl plus choline chloride at a total concentration of 3.2 M), to give final KCl concentrations of 60-160 mM. After a further 5 min the ghosts were resealed by incubating for 60 min at 37 °C. The ghosts were then washed five times in 160 mM choline chloride-10 mM Tris-HCl. Most ghost cells prepared in this manner are relatively impermeable to cations² and capable of active transport³ of Na and K.

To measure incorporation of ³²P-phosphate into ATP, the resealed ghosts, at a final haematocrit of 3%-5%, were incubated at 37 °C in 160 mM NaCl-10 mM Tris-HCl (pH 7.4) in the presence and absence of 0.2 mM ouabain. At timed intervals samples were removed and the ghosts washed twice at 4 °C in 160 mM choline chloride-10 mM Tris-HCl. The packed ghosts were haemolysed in a solution containing 1.4 mM ATP and 2 μM phosphate. (To estimate the initial level of internal K and ³²P-phosphate, ghosts that were resealed but not incubated were treated the same way.) Aliquots were then removed to determine the K and haemoglobin contents. To precipitate the protein in the haemolysate ice-cold trichloroacetic acid was added (final concentration 5%), and the mixture centrifuged.

Table 1 Incorporation of ³²P-orthophosphate into organophosphate by reversal of the Na-K pump in resealed red cell ghosts

Expt	% Of total initial radioactivity in organophosphate		
	Total	Plus ouabain	Ouabain-sensitive
1	2.19	1.73	0.46
2	3.41	2.97	0.44
3	2.05	1.91	0.14
4	2.73	2.17	0.56
5	3.51	3.15	0.36

Ghosts were resealed in a solution containing 160 mM KCl, plus other constituents as given in the text. The ghosts were then incubated for 15 min at 37 °C in 160 mM NaCl-10 mM Tris-HCl, in the presence and absence of 0.2 mM ouabain. The ouabain-sensitive incorporation of ³²P into organophosphate is assumed to represent synthesis of ATP by reversal of the Na-K pump.

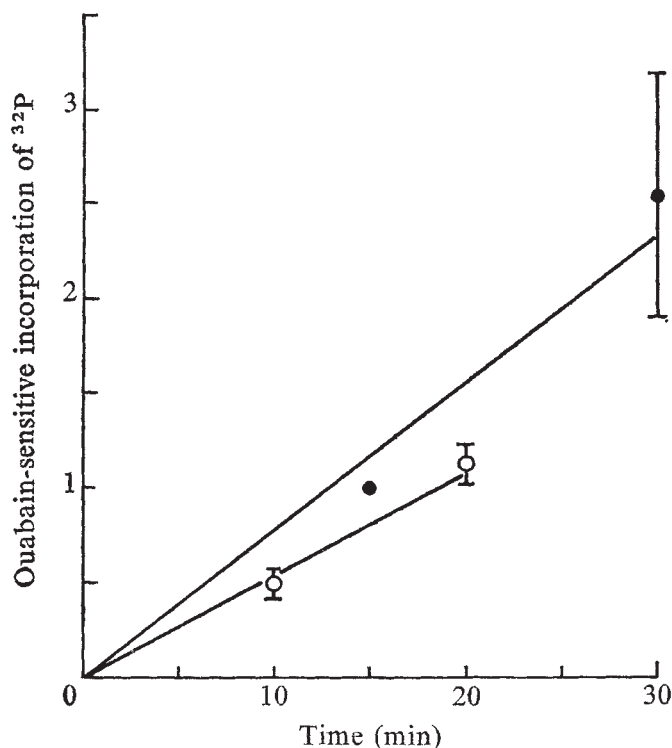


Fig. 1 Time course of incorporation of ^{32}P -orthophosphate into organophosphate by reversal of the Na-K pump. Red cell ghosts were resealed in solutions containing KCl at 160 mM (●) or 80 mM (○), plus other constituents as given in the text. At the times indicated during incubation, samples of the ghosts were taken for determination of ^{32}P -labelled organophosphate and of K concentration. In each experiment, the ouabain-sensitive incorporation of ^{32}P into organophosphate (as a fraction of the initial content of ^{32}P -orthophosphate) was normalised to the value at 15 min for ghosts resealed in 160 mM KCl. Results shown are from four experiments with ghosts resealed in 160 mM KCl (mean final [K] in the ghosts, $95 \text{ mM} \pm 8$, s.e.m.). In three of these experiments ghosts resealed in 80 mM KCl were also used (mean final [K], $54 \text{ mM} \pm 6$). Vertical bars indicate s.e.m. values (except for the value to which the others were normalised). The lines were fitted by eye.

The supernatant material was adjusted to pH 5–6 with Tris, unlabelled phosphate added as carrier, and inorganic phosphate extracted as the phosphomolybdate complex⁴. Radioactivity remaining after the extractions, ' ^{32}P -organophosphate', was then measured by liquid scintillation counting.

In these conditions ghosts resealed in 160 mM KCl and incubated for 15 min in 160 mM NaCl incorporated about 3% of the initial ^{32}P -phosphate into the organophosphate fraction (Table 1). Of this incorporation, about 14% was inhibited by ouabain (0.4% of the initial ^{32}P -phosphate), and this synthesis is assumed to reflect reversal of the Na-K pump^{4,5}. In similar experiments Garrahan and Glynn⁴ found 0.5% of the initial ^{32}P incorporated after 15 min, and 40% of this was ouabain-sensitive (0.2% of the initial ^{32}P).

In a preliminary experiment the extract was separated by ion-exchange chromatography⁴. ouabain-sensitive incorporation into material eluting with authentic ATP was demonstrated, together with a smaller fraction eluting with ADP. Garrahan and Glynn⁴ similarly found that the bulk of incorporated ^{32}P eluted with ATP; the labelling of ADP was attributed to the action of adenylate kinase. In the same experiment ouabain-sensitive influx of ^{22}Na was also demonstrated⁶. This flux could not result from Na-Na exchange since the ghosts, containing a high K content, were virtually free of internal Na.

The time course of ouabain-sensitive incorporation of ^{32}P into organophosphate was nearly linear for 20–30 min, with ghosts resealed in either high or low KCl media

(Fig. 1), although there was considerable error in the estimation of incorporation at 30 min. In these and subsequent experiments the rate of incorporation in each case is normalised to that of ghosts resealed in 160 mM KCl and incubated for 15 min, measured concurrently, defined as 1.0. Normalisation was necessary because of the variation in absolute incorporation between different preparations of ghosts (Table 1). The measured K concentrations were less than those of the resealing media (Fig. 1), but the loss did not occur during the experimental incubation: there was no detectable decrease in K content between 0 and 30 min.

Since it was possible to estimate in 15 min the initial rate of ouabain-sensitive incorporation, the effect of the internal K concentration on this rate was examined (Fig. 2). Although there were again large errors associated with the estimates of incorporation, there was a positive correlation between the measured K concentration within the ghosts and the rate of synthesis. Moreover, these data, replotted in double-reciprocal form (Fig. 2, inset) fit well a linear relationship, giving a $K_{0.5}$ of 0.3 M for K-activation of ATP synthesis through reversal of the Na-K pump.

This $K_{0.5}$ is two to three orders of magnitude greater than the apparent affinity for K at sites inhibiting the binding of ATP^{7,8}, sites that have been interpreted⁹ as representing the internal discharge sites for K. By contrast, this $K_{0.5}$ for pump reversal is only twofold greater than the K_i for K as

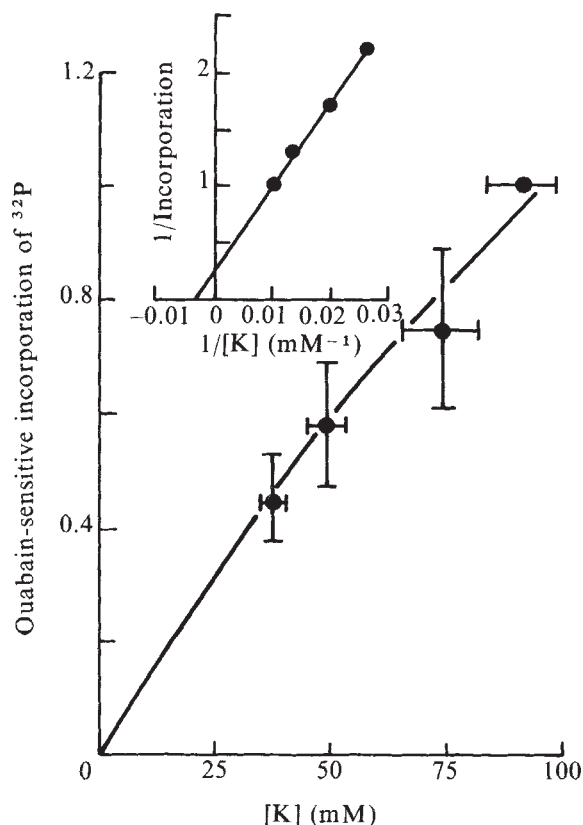


Fig. 2 Incorporation of ^{32}P -orthophosphate into organophosphate in resealed ghosts containing various K concentrations. Ghosts were incubated for 45 min at 37°C . The horizontal and vertical bars indicate s.e.m. values for, respectively, the measured K concentrations in the ghosts and the ouabain-sensitive incorporation of ^{32}P into organophosphate (except for the values at the highest K concentration, to which the others were normalised). The results shown are from four experiments, each with ghosts containing four different K concentrations, plus a fifth experiment with ghosts at two K concentrations. In the inset, these data are replotted in double reciprocal form, and the straight line was fitted by the method of least squares; the calculated $K_{0.5}$ is 297 mM.

a non-competitive inhibitor of K-dependent phosphatase activity of the Na,K-ATPase¹⁰, an effect considered¹⁰ as product-inhibition through occupancy of discharge sites for K.

For K-K exchange through the pump the concentration of internal K for half-maximal activation is only 10 mM (ref. 11), well below the $K_{0.5}$ found here. This discrepancy may reflect mechanistic differences between the two processes; for example, K influx occurs with K-K exchange but not with pump reversal, so if influx becomes rate-limiting before internal K sites (involved in both exchange and reversal) are saturated, then different responses to internal K might be seen. Alternatively, K-K exchange may involve cyclical changes in K sites, rather than a simple shuttling through K channels. Since the K_i for K as a competitor to Na at the internal Na sites is 9 mM (ref. 12), close to the $K_{0.5}$ for K in K-K exchange, it is tempting to consider that efflux during K-K exchange involves those Na sites. Further quantitation of Na-K interactions¹¹ as well as elucidation of the role of inorganic phosphate¹³ will be needed before the nature of the exchange mechanism can be fully resolved.

For the Na-K pump the data can be accommodated in formulations featuring K discharge sites ($K_{0.5}$ approximately 300 mM) that may be cyclically converted to acceptance sites for Na (K_i for K about 9 mM), the latter possibly also serving in K-K exchange as acceptance sites for K ($K_{0.5}$ approximately 10 mM). In any case, our results clearly demonstrate the presence of internal low-affinity sites for K in conditions in which Na acceptance sites would be expected (that is, high nucleotide content and physiological K concentration). The observations thus support formulations¹⁴⁻¹⁶ in which distinct sites and channels for Na and K co-exist, perhaps on different subunits, perhaps with interconversion of these two classes of channels with each pump cycle.

This work was supported by grants from the US PHS and NIH.

J. D. ROBINSON
E. S. HALL

Department of Pharmacology,
State University of New York,
Upstate Medical Center,
766 Irving Avenue,
Syracuse, New York 13210

P. B. DUNHAM

Department of Biology,
Syracuse University,
Syracuse, New York 13210

Analgesic activity of lipotropin C fragment depends on carboxyl terminal tetrapeptide

A SERIES of peptides with morphine-like activity has been identified in extracts of pituitary¹⁻³ and brain⁴⁻⁷. With the exception of Leu-enkephalin, all those characterised represent some fraction of the 31-residue C fragment—a specific activation product of lipotropin^{1,8}. Several attempts have been made to define the receptor requirements of opiate peptides^{9,10} and to make potent and stable agonists^{5,11,12}, but essentially all the studies have been based on the easily synthesised NH₂-terminal pentapeptide of C fragment, Met-enkephalin. Unlike the enkephalins, C fragment has a high potency in its central actions¹³⁻¹⁶, *in vivo* and *in vitro*, and it is relatively stable to brain peptidases¹⁷⁻¹⁹. C fragment is the only naturally occurring peptide to possess strong analgesic properties and have a long lasting action²⁰. It is clear that specific regions of the molecular structure, not present in γ and α endorphin or Met-enkephalin, have a role in the expression of its central activity. We present here evidence that the COOH-terminal tetrapeptide of C fragment, Lys-Lys-Gly-Gln, is responsible for the unique affinity of C fragment for brain opiate receptors and that the presence of all four residues is essential for the high analgesic potency.

Lipotropin, C fragment (61-91) and C' fragment (61-87) were isolated from porcine pituitary¹. The 61-89 peptide and the ϵ -N-pentacitraconyl derivative of C fragment were prepared as described in Table 1. Opiate binding assays were carried out by measuring the ability of peptides to reduce the specific binding of ³H-naloxone and ³H-dihydromorphine to opiate receptors in washed synaptosomal membranes from rat brain. Analgesic potencies were determined by a tail flick assay using rats with indwelling cannulae²¹.

The affinity of C fragment (lipotropin 61-91) for brain opiate receptors, assessed by displacement of ³H-naloxone, was decreased by removal of the COOH-terminal glutamine and glycine residues (Table 1). C' fragment (61-87), which lacks two lysine residues in addition to glutamine and glycine, exhibited a much lower affinity; its ability to displace ³H-naloxone was 40 times less than that of C fragment.

Table 1 Binding of C fragment and derivatives to brain synaptosomal membranes

Peptide	Concentration required to displace 50% of bound opiate	
	³ H-naloxone (10 ⁸ M)	³ H-dihydromorphine (10 ⁸ M)
C fragment	2.6	2.2
C fragment (61-89)	7 ± 2 (4)	2.2 ± 0.5 (4)
C' fragment (61-87)	100	42
[CT ₅] C fragment	4,000	710
Acid-treated [CT ₅] C fragment	3.0	2.5

Desglutamine desglycine C fragment (61-89) was obtained by prolonged carboxypeptidase A digestion of citraconylated C fragment. The product was isolated by gel filtration on Sephadex G50 in 200 mM N-ethylmorpholine acetate at pH 8.5. ϵ -N-pentacitraconyl C fragment ([CT₅] C fragment) was prepared by trypsin digestion of citraconylated porcine lipotropin. The peptide was purified by gel filtration on Sephadex G50 in 20 mM Tris-HCl at pH 8.5 and by ion-exchange chromatography on DEAE-Sephadex A25 at pH 8.5. Citraconyl groups were removed from peptides by incubation with 100 mM pyridine acetate at pH 4.0 for 24 h at 37 °C. Volatile buffers were removed by evaporation in a vacuum and by desalting on Sephadex G25. Peptide purity was established by amino acid analysis and NH₂- and COOH-terminal determinations. Opiate binding assays were performed using displacement of radiolabelled alkaloids from brain synaptosomal membrane preparations as a measure of the relative affinity of a given peptide⁹. The percentage error of the mean of a determination was generally better than 3%. The standard deviations shown include results obtained from two preparations of the 61-89 peptide.

Received 9 June; accepted 26 July 1977.

- Glynn, I. M. & Karlish, S. J. D. *A. Rev. Physiol.* **37**, 13-55 (1975).
- Bodemann, H. & Passow, H. *J. Membr. Biol.* **8**, 1-26 (1972).
- Bodemann, H. & Hoffman, J. F. *J. gen. Physiol.* **67**, 497-525 (1976).
- Garrahan, P. J. & Glynn, I. M. *J. Physiol., Lond.* **192**, 237-256 (1967).
- Glynn, I. M. & Lew, V. L. *J. Physiol., Lond.* **207**, 393-402 (1970).
- Sachs, J. R., Ellory, J. C., Kropp, D. L., Dunham, P. B. & Hoffman, J. F. *J. gen. Physiol.* **63**, 389-414 (1974).
- Hegyvary, C. & Post, R. L. *J. biol. Chem.* **246**, 5234-5240 (1971).
- Robinson, J. D. *Biochim. biophys. Acta* **397**, 194-206 (1975).
- Post, R. L., Kume, S. & Rogers, F. N. in *Mechanisms in Bioenergetics* (eds Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E. & Siliprandi, N.) (Academic, New York, 1973).
- Robinson, J. D. *Biochim. biophys. Acta* **384**, 250-264 (1975).
- Simons, T. J. B. *J. Physiol., Lond.* **237**, 123-155 (1974).
- Garay, R. P. & Garrahan, P. J. *J. Physiol., Lond.* **231**, 297-325 (1973).
- Glynn, I. M., Lew, V. L. & Lüthi, U. *J. Physiol., Lond.* **207**, 371-391 (1970).
- Robinson, J. D. *Archs Biochem. Biophys.* **156**, 232-243 (1973).
- Garrahan, P. J. & Garay, R. P. *Curr. Top. Membr. Transp.* **8**, 29-97 (1976).
- Robinson, J. D. *Biochim. biophys. Acta* **482**, 427-437 (1977).

Similar results were obtained when displacement of ^3H -dihydromorphine was used as an index of receptor binding, but the decrease in affinity associated with removal of the COOH-terminal residues from C fragment was less marked. Thus, desglutamine desglycine C fragment (61–89) exhibited a receptor affinity comparable to C fragment and removal of the two lysine residues reduced affinity 19-fold.

The results indicate an important role for the paired lysine residues, and to a lesser extent glycine and glutamine at the COOH-terminus, for the binding of C fragment to brain opiate receptors. A requirement for positive charges on the lysine residues was suggested by study of the binding properties of a pentacitraconyl derivative of C fragment, in which the five lysine amino groups were masked by anionic substitution. The affinity of the citraconyl derivative, measured by displacement of naloxone, was three orders of magnitude less than the affinity of C fragment.

It was notable that the potency of ϵ -N-pentacitraconyl C fragment in displacing ^3H -dihydromorphine was greater than its potency in displacing ^3H -naloxone (Table 1) and in this respect the citraconylated derivative resembles Met-enkephalin. This is consistent with the finding that modifications in the COOH-terminal region of C fragment have more effect on the inhibition of naloxone binding than on the inhibition of morphine binding.

The analgesic activities exhibited by C fragment, the 61–89 peptide and C' fragment reflected their abilities to displace ^3H -naloxone in the binding assay. Removal of the glutamine and glycine residues from the COOH-terminus of C fragment reduced the analgesic potency (Fig. 1). The effect of removing the two lysine residues, shown in the properties of C' fragment, was to cause a severe loss of potency; the 61–87 peptide possessed approximately 1/500th the activity of C fragment.

Although the potency of C' fragment was low, its analgesic action persisted for several hours. In contrast, the weak analgesic action of Met-enkephalin is transient^{22,23}. It is clear that the duration of analgesia, and not the potency, is related to *in vivo* stability. Met-enkephalin, for example, undergoes rapid degradation in the presence of brain homogenates²⁴ whereas C fragment and C' fragment are comparatively stable¹⁷. The long peptides seem to possess preferred conformations which protect the NH₂-terminal region from enzymatic attack²⁵. In particular it is notable that C' fragment resembles C fragment in having a high stability to

attack by aminopeptidases, which would indicate that the two peptides possess similar conformations in solution. Our results suggest that the four residues at the COOH-terminus of C fragment contribute to receptor affinity by participating in a specific interaction within the peptide-receptor complex and not by stabilising an intrinsic conformation of the peptide molecule.

The COOH-terminal residues of C fragment, Lys-Lys-Gly-Gln, are highly resistant to the action of carboxypeptidases in brain¹⁸. Furthermore, loss of all four residues leads to a large reduction in analgesic potency and removal of additional residues has little further effect on binding affinity⁹ or analgesia²⁰. Thus, the COOH-terminal region of C fragment fulfils a dual function: it confers stability on the peptide against proteolysis and it provides a structure important for receptor affinity.

We thank D. E. Massey and Ann Smith for technical assistance.

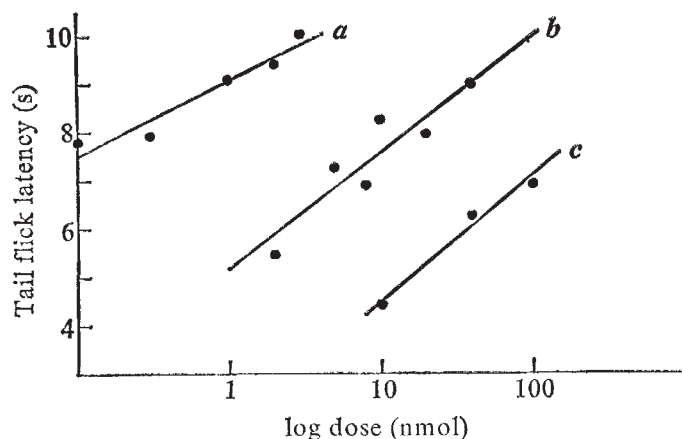
M. J. GEISOW
J. F. W. DEAKIN
J. O. DOSTROVSKY
D. G. SMYTH

National Institute for Medical Research,
Mill Hill,
London NW7, UK

Received 21 June; accepted 18 July 1977.

- Bradbury, A. F., Smyth, D. G. & Snell, C. R. in *Peptides: Chemistry, Structure and Biology* (eds Walter, R. & Meinhofer, J.) 609–615 (Science, Ann Arbor, 1975).
- Bradbury, A. F., Smyth, D. G. & Snell, C. R. in *Polypeptide Hormones: Molecular and Cellular Aspects*, CIBA Found. Symp. No. 41, 61–75 (Elsevier-North Holland, Amsterdam 1976).
- Graf, L., Barat, E. & Patty, A. *Acta Biochem. biophys. Acad. Sci. Hung.* **11**, 111 (1976).
- Hughes, J. *et al. Nature* **258**, 577–579 (1975).
- Bradbury, A. F., Feldberg, W. S., Smyth, D. G. & Snell, C. R. in *Opiates and Endogenous Opioid Peptides* (ed. Kosterlitz, H. W.) 9–17 (Elsevier-North Holland, Amsterdam, 1976).
- Simantov, R. & Snyder, S. H. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2515–2519 (1976).
- Guillemin, R., Ling, N. & Burgus, R. C. *r. hebdom. Séanc. Acad. Sci., Paris* **282**, 783–785 (1976).
- Bradbury, A. F., Smyth, D. G. & Snell, C. R. *Biochem. biophys. Res. Commun.* **69**, 950–956 (1976).
- Bradbury, A. F., Smyth, D. G., Snell, C. R., Birdsall, N. J. M. & Hulme, E. C. *Nature* **260**, 793–795 (1976).
- Lazarus, L. H., Ling, N. & Guillemin, R. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2156–2159 (1976).
- Pert, C. B., Bowie, D. L., Fong, F. T. W. & Chang, J. K. in *Opiates and Endogenous Opioid Peptides* (ed. Kosterlitz, H. W.) 79–86 (Elsevier-North Holland, Amsterdam, 1976).
- Coy, D. H. *et al. Biochem. biophys. Res. Commun.* **73**, 632–638 (1976).
- Feldberg, W. S. & Smyth, D. G. *J. Physiol., Lond.* **260**, 30–31P (1976); **265**, 25–27P (1977).
- Loh, H. H., Tseng, L. F., Wei, E. & Li, C. H. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2895–2898 (1976).
- Bradbury, A. F., Smyth, D. G., Snell, C. R., Deakin, J. F. W. & Wendlandt, S. *Biochem. biophys. Res. Commun.* **74**, 748–754 (1977).
- Gent, J. P., Smyth, D. G., Snell, C. R. & Wolstencroft, J. H. *J. Pharmac.* (in the press).
- Austen, B. M. & Smyth, D. G. *Biochem. biophys. Res. Commun.* **76**, 477–482 (1977).
- Geisow, M. J. & Smyth, D. G. *Biochem. biophys. Res. Commun.* **75**, 625–629 (1977).
- Austen, B. M., Smyth, D. G. & Snell, C. R. *Nature* (submitted for publication).
- Feldberg, W. S. & Smyth, D. G. *Br. J. Pharmac.* **60**, 445–454 (1977).
- d'Amour, F. E. & Smith, D. L. *J. Pharmac. exp. Ther.* **72**, 74–79 (1941).
- Belluzi, J. D. *et al. Nature* **260**, 625–626 (1976).
- Büscher, H. H. *et al. Nature* **261**, 423–425 (1976).
- Hambrook, J. M., Morgan, B. A., Rance, M. J. & Smith, C. F. C. *Nature* **262**, 782–783 (1976).
- Austen, B. M. & Smyth, D. G. *Biochem. biophys. Res. Commun.* **77**, 86–94 (1977).

Fig. 1 Log dose-response curves for analgesia produced by intraventricular injection of peptides in the rat. *a*, C fragment 61–91; *b*, 61–89 peptide; *c*, C' fragment (61–87). Peptides were dissolved in 0.9% saline and injected in a volume of 5 μl through indwelling lateral ventricular cannulae. Tail-flick latencies were measured before and at intervals up to 3 h subsequent to administration of the peptides. The ordinate values give the tail-flick latency at 30 min after injection. There were no significant differences between groups in baseline latency (2–4 s).



Proinsulin conversion to desalanyl insulin by α_2 -macroglobulin-bound trypsin

HARPEL and Mosesson¹ observed that α_2 -macroglobulin-bound trypsin hydrolysed low molecular weight ester and amide substrates at rates comparable with those of free protease, but degradation of high molecular weight proteins was markedly inhibited. To account for this finding, Barrett and Starkey² introduced the novel concept of steric entrapment of endopeptidases by α_2 -macroglobulin. Neurath and Walsh³ have recently reviewed the role of proteolytic enzymes in the conversion of a variety of enzymes, hormones, and other physiologically active proteins from inactive precursors to active forms by limited

proteolysis. Many of these physiologically active proteins exist in the bloodstream in both precursor and active forms. We report here that proinsulin, a large polypeptide of 9,000 molecular weight, is rapidly hydrolysed by α_2 -macroglobulin-bound trypsin. This finding suggests that proteases bound to α_2 -macroglobulin may be involved in limited proteolytic cleavage associated with activation of precursor molecules as well as the degradation of biologically active polypeptides in the circulation.

Proinsulin was chosen as a model for investigation of the hydrolysis of polypeptide hormones by α_2 -macroglobulin-bound proteases because it is a polypeptide of known sequence⁴ and representative size. Tryptic conversion of proinsulin to desalanyl insulin proceeds by way of two intermediate forms of proinsulin (insulin-Arg and insulin-Arg-Arg) with specific cleavages in the C-peptide region^{5,6}. Proinsulin, the intermediate forms, and desalanyl insulin can be resolved by polyacrylamide gel electrophoresis at pH 8.9 (ref. 4).

The digestion of porcine proinsulin (gift of Dr R. E. Chance, Lilly) was performed at 37 °C with an enzyme: substrate ratio of 1:200. A control incubation of proinsulin with α_2 -macroglobulin was included to assess the stability of proinsulin at 37 °C as well as the possible presence of endogenous protease-inhibitor complexes in the purified preparation of α_2 -macroglobulin. The gel electrophoresis pattern for digestion of proinsulin by α_2 -macroglobulin-bound trypsin or free enzyme is shown in Fig. 1. Chance *et al.*⁴ have shown that free trypsin rapidly digests proinsulin by way of two intermediate products to yield desalanyl insulin, which cannot be resolved from insulin in this gel electrophoresis system⁷. The intermediate forms seen in lanes 7–10 of Fig. 1 are presumed to be insulin-Arg and insulin-

Arg-Arg on the basis of previous investigations of the tryptic hydrolysis of proinsulin^{4–7}. The lighter appearance of the band for desalanyl insulin is due to some further digestion of trypsin, as well as to more rapid diffusion of smaller hydrolysis products from the gel, as can be seen by comparing the stain intensity of proinsulin (lane 1, 25 μ g) with that of insulin (lane 11, 50 μ g). By comparison of densitometer tracings of the proinsulin band at different times of hydrolysis, we estimate that proinsulin is digested by α_2 -macroglobulin-bound trypsin at approximately 10–15% of the rate of hydrolysis by the free enzyme.

Chance *et al.*⁴ have previously observed that trypsin hydrolyses proinsulin more rapidly than insulin or desalanyl insulin. The pattern of tryptic hydrolysis of proinsulin is also altered by binding of the enzyme to α_2 -macroglobulin (see Fig. 1). An intermediate product is apparent which is not present in detectable levels when proinsulin is digested with free trypsin. In addition, the major intermediate during digestion with trypsin does not accumulate to such a large extent during digestion with α_2 -macroglobulin-bound trypsin. This finding may reflect a more rapid diffusion of desalanyl insulin (molecular weight (MW) 5,600) and the intermediate products (MWs 6,050 and 5,900) compared with proinsulin (MW 9,000) into the enzyme-inhibitor complex.

Inactivation of the small peptide hormones angiotensin (MW 1,045) and lysine vasopressin (MW 1,116) by α_2 -macroglobulin-bound trypsin was reported previously⁸. Inactivation of these hormones involves proteolytic cleavage of two residues from the N terminal of the former peptide, while the latter is inactivated by cleavage of a C-terminal lysine amide, both of which might represent partial diffusion of a small peptide chain into the inhibitor-enzyme complex. In addition, plasmin bound to α_2 -macroglobulin has been

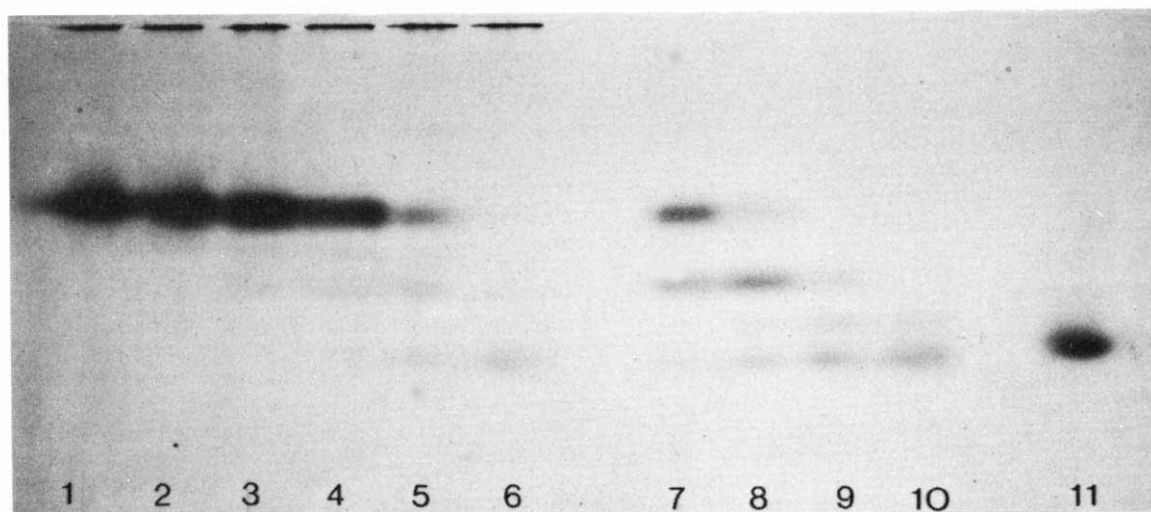


Fig. 1 Conversion of proinsulin to desalanyl insulin by α_2 -macroglobulin-bound trypsin. Lanes 1 and 2, proinsulin and α_2 -macroglobulin at 0 and 120 min; lanes 3–6, α_2 -macroglobulin-bound trypsin digestion of proinsulin at 15, 30, 60 and 120 min; lanes 7–10, trypsin digestion of proinsulin at 7, 15, 30 and 60 min, lane 11, insulin standard. Human α_2 -macroglobulin was purified from fresh plasma by DEAE-cellulose chromatography and gel filtration on Sephadex G-150 and Ultrogel ACA 22, a combination Agarose-polyacrylamide gel filtration resin (LKB). Bovine trypsin (Worthington) was treated with 1-tosylamido-2-phenylethylchloromethyl ketone to eliminate contaminating chymotryptic activity. Trypsin was pre-incubated with a 1.5-fold molar excess of α_2 -macroglobulin to produce an enzyme-inhibitor complex as follows: a 100- μ l aliquot of trypsin (0.1 mg ml⁻¹) was incubated with 300 μ l of α_2 -macroglobulin (1.2 mg ml⁻¹) in 40 mM Tris-HCl (pH 9.5) for 30 min at 37 °C. A control solution of enzyme and buffer was incubated in identical conditions. The extent of inhibition was measured using Remazol brilliant blue dyed hide powder (RBB-hide) as substrate as previously described¹⁰. Using this method, 0.1% of the original trypsin activity would have been detected. In the inhibition conditions used, however, no trypsin activity could be measured at 30 min, 2 h, or 24 h of incubation at 37 °C, while the control trypsin solution retained at least 90% activity after 24 h of incubation. Digestion mixtures consisted of a 40- μ l aliquot of the preincubation mixture of trypsin and α_2 -macroglobulin or trypsin alone with 100 μ l of proinsulin (2 mg ml⁻¹ in 40 mM Tris-HCl, 20 mM CaCl₂, pH 9.5). Sample aliquots (20 μ l) from the digestion mixtures were added to 16 mg of solid urea to form an 8 M urea solution and stored at -60 °C until electrophoresis. Polyacrylamide slab gel electrophoresis was performed at pH 8.9 using a modification⁷ of the original procedure described by Davis¹¹ in which 7 M urea was added to the resolving and stacking gels to improve resolution. The resolving gel contained 15% acrylamide with 0.4% *N,N'*-methylene-bis-acrylamide. Electrophoresis was performed for 16 h at 75 V in a BioRad Model 220 slab gel electrophoresis apparatus. Gel slabs were stained for 1 h in 0.1% amido black in 10% acetic acid, 20% ethyl alcohol and rapidly destained in 10% acetic acid to minimise diffusion of the peptide products from the gel. Using this procedure, the intensity of the bands remained constant for approximately 24 h.

shown to hydrolyse fibrinogen by degrading the A α -chain at a rate approximately 0.1% of that of the free enzyme¹. Our findings suggest that the inhibitor-enzyme matrix is substantially permeable to diffusion of peptides of moderate size. The tryptic cleavage position in proinsulin which produce desanyl insulin are at Arg-33 and Arg-63 in a polypeptide chain of 80 amino acid residues. Thus it seems that α_2 -macroglobulin-bound trypsin at approximately 10–15% variety of peptide hormones with molecular weights smaller than or comparable with that of proinsulin.

We have recently reported the presence of a pancreatic endopeptidase (elastase 2) in human blood and the elevation of this enzyme in sera of patients with acute pancreatic inflammation⁹. Pancreatic cationic trypsin has also been detected in human serum in this laboratory and will be the subject of a future communication. We suggest that some of the pathophysiological events associated with acute pancreatic inflammation may be due to hydrolysis of polypeptide hormones by circulating complexes of pancreatic proteases bound to α -macroglobulin.

This work was supported by the Medical Research Service of the Veterans Administration.

COREY LARGMAN
JANICE H. JOHNSON
JAMES W. BRODRICK
MICHAEL C. GEOKAS

Enzymology Research Laboratory,
Veterans Administration Hospital,
Martinez, California 94553, and
Department of Medicine,
University of California School of Medicine,
Davis, California 95616

Received 4 May; accepted 11 July 1977.

- ¹ Harpel, P. C. & Mosesson, M. W. *J. clin. Invest* **52**, 2175–2184 (1973).
- ² Barrett, A. J. & Starkey, P. M. *Biochem. J.* **133**, 709–724 (1973).
- ³ Neurath, H. & Walsh, K. A. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3825–3832 (1976).
- ⁴ Chance, R. E., Ellis, R. M. & Bromer, W. W. *Science* **161**, 165–167 (1968).
- ⁵ Chance, R. E. *Diabetes: Proc. Seventh Cong. Int. Diabetes Fed., Buenos Aires, 1970* (eds Rodriguez, R. R. & Vallance-Owen, J.) 292–305 (Excerpta Medica, Amsterdam, 1971).
- ⁶ Steiner, D. F., Hallund, O., Rubenstein, A., Cho, S. & Bayliss, C. *Diabetes* **17**, 725–736 (1968).
- ⁷ Yip, C. C. *Proc. natn. Acad. Sci. U.S.A.* **68**, 1312–1315 (1971).
- ⁸ Rinderknecht, H. & Geokas, M. C. *Biochim. biophys. Acta* **295**, 233–244 (1973).
- ⁹ Geokas, M. C., Brodrick, J. W., Johnson, J. H. & Largman, C. *J. biol. Chem.* **252**, 61–67 (1977).
- ¹⁰ Rinderknecht, H., Silverman, O., Geokas, M. C. & Haverback, B. J. *Clin. chim. Acta* **28**, 239–246 (1970).
- ¹¹ Davis, B. *Ann. N.Y. Acad. Sci.* **121**, 404–427 (1964).

Structural analysis of purified platelet-activating factor by lipases

PLATELET-activating factor (PAF) is a newly described mediator of anaphylaxis which has been implicated in the deposition of immune complexes in acute serum sickness in rabbits^{1–4}. It is released by leukocytes and tissues, most probably by the basophils or mastocytes from various mammalian species, under the influence of various substances known to activate these cells^{5,6}. The study of the chemical nature of the compound responsible for the PAF activity led us to the preparation of large amounts of PAF from hog leukocytes⁵. Several fractionation steps of the crude material yielded a highly active chloroform-soluble fraction. We usually started these procedures with 100 l of hog blood which yielded 100 l of PAF, the biological activity of which could be detected at the 1- μ l level. Nevertheless, we have not yet obtained sufficient homogeneous material to apply the current methods for structural analysis (mass spectrometry, magnetic nuclear resonance, hydrolysis reactions and so on). We therefore used different lipases to get some insight into the structure of PAF. We present here the results of these experiments which suggest that PAF is a 1-lyso-glycerophospholipid.

PAF was obtained by procedures described previously^{3,5} modified for use on bulk quantities of hog blood. Throughout the purification steps, PAF activity was detected by aggregation of rabbit platelets⁵, in the presence of 10 μ M indomethacin⁷. Briefly, leukocytes from 100 l of blood were prepared by differential centrifugations followed by sedimentation in 2.5% gelatin in Tris-buffered saline (TBS), pH 7.4. (TBS contained Tris (hydroxymethyl)-amino-methane, 1×10^{-2} M; NaCl, 1.37×10^{-1} M; pH adjusted to 7.4 or 8 with HCl. Tyrode's contained TBS plus MgCl₂, 1×10^{-3} M; CaCl₂, 1×10^{-3} M; KCl, 2.6×10^{-3} M.) PAF affixed to bovine serum albumin (BSA) was obtained by overnight incubation of the leukocytes in Tris-buffered Tyrode's at pH 9.5, containing 0.25% BSA. PAF-BSA (100 l) was exchanged in batch on to QAE-Sephadex-A50 at pH 9.5; PAF was then eluted by washing the beads with 80% and 100% ethanol. After concentration under vacuum, the ethanol-water residue was extracted with chloroform. The lipid (3–4 g) thus obtained was chromatographed on a silicic acid column developed with chloroform, chloroform-methanol mixtures, and pure methanol. PAF-active, phospholipid-rich (major phospholipids were phosphatidylcholine, sphingomyelin and lysophosphatidylcholine, as shown by thin layer chromatography (TLC) and chemical ionisation mass spectrometry), fractions eluted with pure methanol, were further purified by high pressure liquid chromatography on a silicic acid column (Varian, Micropak Si-5) with chloroform-methanol-water as solvent. The active fraction (1–2 mg of lipids) was eluted between sphingomyelin and lysophosphatidylcholine; it migrated between these phospholipids on silica gel TLC, with a solvent containing by volume 100 : 5 : 1 of CHCl₃ : CH₃OH : H₂O. (Development was by use of iodine, Dittmer⁸ or Dragendorff reagents.) Final recovery was 25% of starting activity.

Three preparations of PAF were treated with the lipases: (1) crude PAF-BSA, dialysed overnight against TBS; (2) the most active fraction obtained after the first silicic acid chromatography, which exhibited three or four spots on TLC; and (3), the purest fraction described above.

The lipases used in this study were lipase from *Rhizopus arrhizus*, phospholipases A2 from either porcine pancreas or *Crotalus*⁹, phospholipases C from *Bacillus cereus*⁹, and D from cabbage¹⁰. Dr Zwaal (from Van Deenen's Laboratory, Utrecht) kindly provided purified phospholipase A2 from *Naja naja* and *Crotalus* and a phospholipase C from *Staphylococcus aureus* specific for sphingomyelin (sphingomyelinase)¹¹.

The conditions and the results of the treatment of PAF with lipases are shown in Table 1. The phospholipases A2 (from various origins) and the phospholipases C and D completely abolished the ability of PAF (preparations 2 and 3) to aggregate rabbit platelets. In contrast, the lipase from *R. arrhizus*, which hydrolyses exclusively the fatty acid ester bond at the 1-position of phosphoglycerides^{12,13}, and sphingomyelinase C, were totally ineffective in this respect. It should be noted that PAF-BSA (preparation 1) was insensitive to the action of phospholipases A2, but fully sensitive to phospholipases C and D. Controls for optimal conditions for the action of lipases were hydrolysis of phosphatidylcholine or, for sphingomyelinase, sphingomyelin, followed by TLC. We also verified that PAF treated with phospholipases A2, C and D, in the presence of EDTA, not only retained its full activity, but also migrated on TLC with the same *R_f* as untreated PAF. Finally, the purest PAF fraction (preparation 3) was first treated with sphingomyelinase C, then exposed to lipase from *R. arrhizus* as well as to phospholipases A2 and C. The results were the same as those reported above, indicating that treatment with sphingomyelinase, besides not affecting PAF activity, does not seem to modify the structure of the molecule.

The lack of effect of lipase from *R. arrhizus* and the sensitivity of PAF to phospholipases A2, C and D seems to

Table 1 Action of lipases on PAF activity

Lipases (concentrations per ml)	Medium	PAF activity (units ml ⁻¹)
Lipase (<i>R. arrhizus</i>)* 0.1 mg	Borate 0.1 M pH 6.5 CaCl ₂ 10 mM Deoxycholate 1 mg ml ⁻¹ BSA 0.4 mg ml ⁻¹	148
A2 (Porcine pancreas* or <i>Crotalus</i>) 0.1 mg	TBS pH 8 CaCl ₂ 10 mM	0†
A2 (<i>N. naja</i>), purified† 0.05 unit	TBS pH 8 CaCl ₂ 10 mM	0‡
A2 (<i>Crotalus</i>), purified† 0.5 unit	TBS pH 8 CaCl ₂ 10 mM	0‡
C (<i>B. cereus</i>)* 0.001 mg	TBS pH 8 CaCl ₂ 10 mM	0‡
D (cabbage)* 0.01 mg	Acetate buffer 0.1 M pH 5.6 CaCl ₂ 50 mM	0‡
C (<i>S. aureus</i>)† Sphingomyelinase, purified 4 units	TBS pH 8 CaCl ₂ 10 mM MgCl ₂ 0.25 mM	136

Experimental conditions were 15 min incubation at 37 °C except for lipase from *R. arrhizus* which was incubated for 18 h at 30 °C with constant agitation and phospholipase D, incubated 1 h at 20 °C. These experiments were repeated five times with identical results. PAF activity is expressed in units, as defined in refs 3 and 6. Starting solution of PAF contained 140 units ml⁻¹.

* From Boehringer-Mannheim.

† Gift from Dr Zwaal, Utrecht, The Netherlands.

‡ Same experiments done in the presence of 0.01 M EDTA: PAF activity was identical to that of starting solution.

favour PAF being a 1-lyso (or 2-acyl)-glycerophospholipid. Methylation by diazomethane for 10 min at room temperature¹⁴ did not affect the activity and the *R_f* of PAF, a result which suggests the presence of a choline polar head group. We therefore attempted to produce a PAF-like substance by hydrolysis of a variety of commercially available phospholipids with lipase from *R. arrhizus*, in order to obtain 1-lyso derivatives. We occasionally detected an aggregating activity shown to be due to production of arachidonic acid by its *R_f* on TLC and its inhibition by indomethacin. No PAF-like substance was obtained.

2-Acyl-glycerophospholipids are known to be unstable, and to isomerise to 1-acyl compounds¹⁵. This rearrangement, which occurs to some extent during chromatographic separation of the phospholipid, could account for the relatively low yield of our procedures, and could also represent an inactivation process of a potentially harmful mediator. The PAF-containing fraction, at the present state of purification, seems to be associated, in the chromatographic systems used, with sphingomyelin and lyso-phosphatidylcholine. The complete structure of PAF will be definitively known when more of the pure compound becomes available, making a more direct approach possible. But as the specificity for phospholipids of the enzymes used in this study is well established^{12,13} our present data leads us to postulate a phospholipidic structure for PAF, unique amongst the mediators of anaphylaxis.

This work was supported by contract 75-7-0942 from the Délégation Générale à la Recherche Scientifique et Technique and ATP 76-63 from INSERM.

J. BENVENISTE
J. P. LE COUEDIC

INSERM U25, Hôpital Necker,
161 rue de Sèvres, 75730 Paris Cedex 15

J. POLONSKY
M. TENCE

Institut de Chimie des Substances Naturelles,
CNRS, 91190 Gif-sur-Yvette, France

Received 9 March; accepted 14 July 1977.

*To whom correspondence should be addressed.

- 1 Siraganian, R. P. & Osler, A. G. *J. Immun.* **106**, 1244–1251 (1971).
- 2 Henson, P. M. & Cochrane, C. G. *J. exp. Med.* **133**, 554–571 (1971).
- 3 Benveniste, J., Henson, P. M. & Cochrane, C. G. *J. exp. Med.* **136**, 1356–1377 (1972).
- 4 Benveniste, J., Egido, J. & Gutierrez-Millet, V. *Clin. exp. Immun.* **26**, 449–456 (1976).
- 5 Benveniste, J. *Nature* **249**, 581–583 (1974).
- 6 Camussi, J., Mencia-Huerta, J. M. & Benveniste, J. *Immunology* (in the press).
- 7 Cazenave, J. P., Benveniste, J. & Fraser-Mustard, J. *Fedn Proc.* **36**, 454 (1977).
- 8 Dittmer, J. C. & Lester, R. L. *J. Lipid Res.* **5**, 126–127 (1964).
- 9 *Meth. Enzym.* **32B**, 147–154 (1974).
- 10 *Meth. Enzym.* **35B**, 226 (1975).
- 11 Zwaal, R. F. A., Roelofs, B., Comfurius, P. & Van Deenen, L. L. M. *Biochim. biophys. Acta* **406**, 83–96 (1975).
- 12 Slotboom, A. J., De Haas, G. H., Bensen, P. P. M., Burbach-Westerhuis, G. J. & Van Deenen, L. L. M. *Chem. Phys. Lipids* **4**, 15–29 (1970).
- 13 Semeriva, M., Benzonana, G. & Desnuelle, P. *Bull. Soc. Chim. Biol.* **49**, 71 (1967).
- 14 Crone, H. D. *Biochim. biophys. Acta* **84**, 665–680 (1964).
- 15 De Haas, A. J. & Van Deenen, L. L. M. *Biochim. biophys. Acta* **106**, 315–326 (1965).

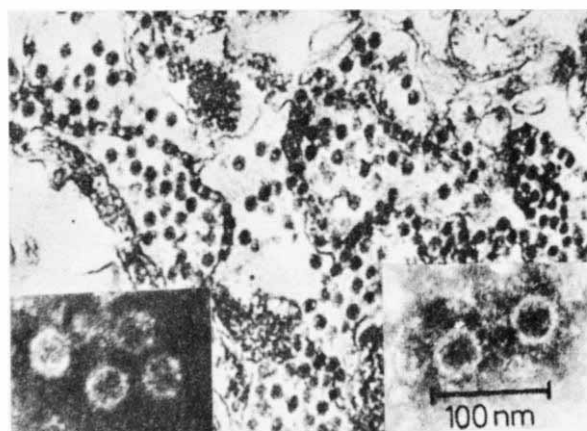
New oncogenic papova virus from primate cells

THE taxonomic group of papova viruses comprises a number of small icosahedral virus species, all isolated from mammals. The members of this group, such as papilloma, polyoma and the so-called vacuolating Simian virus 40 (SV40) contain double-stranded circular closed DNA. All are oncogenic, capable of transforming cells *in vitro*. Some viruses of this group which are called BK¹ and JC² have been isolated from human patients—BK-virus is apparently widespread, as more than 80% of the human population harbour antibodies against this virus³. We describe here a new member of the papova virus group, designated HD-virus, which we have isolated in our laboratory from primate cells (VERO-cells—a permanent line derived from *Cercopithecus aethiops* monkey kidney cells⁴—which have been routinely passaged for several years in our laboratory). HD-virus is capable of rapidly transforming cells *in vitro*.

Electron micrographs of ultra-thin sections of the cells (Fig. 1) reveal particles that correspond in size and morphology to SV40, although their diameter seems slightly smaller.

Incubation of growing VERO-cells (1 d after seeding) with either ³H-thymidine or ³²P-orthophosphate leads to the synthesis of radioactively labelled superhelical HD-viral DNA which can be selectively isolated and purified in a dye buoyant density gradient⁵. Stationary cells (6 d after seeding) reveal, in contrast, a smaller amount of HD-DNA (one-fifteenth of the amount obtained 1 d after seeding of the cells). The size of HD-DNA was estimated by comparison with the electrophoretic mobilities of SV40 and BK-DNAs in Agarose gels to be equivalent to 5,100 base pairs (molecular weight 3.1 × 10⁶ compared with 3.6 × 10⁶ for SV40 DNA). Thus, the HD-DNA is smaller than SV40

Fig. 1 Electron micrograph of an infected VERO-cell. Inserts: micrographs of negatively stained virions released from infected cells by freezing and thawing and concentrated by centrifugation.



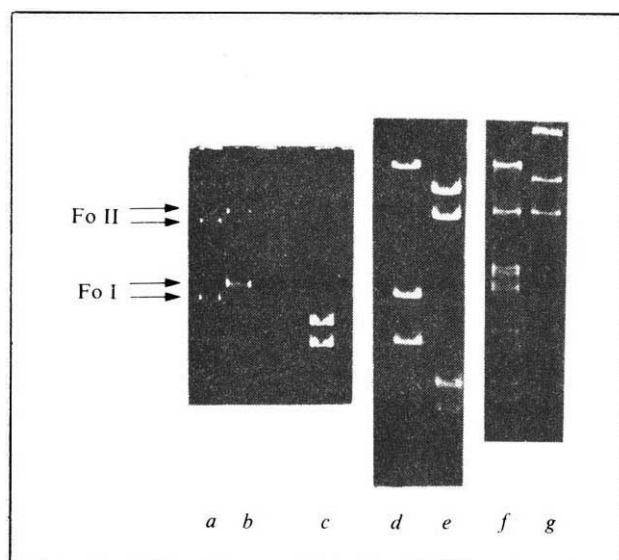


Fig. 2 Gel electrophoresis of DNA of HD, BK and SV40 virus. HD (a) and SV40 (b) form I and form II DNAs are shown in a and b. Fragments produced by digesting HD-DNA with *EcoRI* are depicted in c. A mixture of *HindIII* + III was used to digest HD (d, g) BK- (e) and SV40-DNA (f). After electrophoresis the DNA was stained with ethidium bromide and photographs were taken under ultra violet light (a-e Agarose gel; f, g, polyacrylamide gel).

DNA by 14% (Fig. 2a, b). The susceptibility to various restriction endonucleases also distinguishes the HD genome from other papova virus genomes such as BK (Fig. 2e) and SV40 (Fig. 2f). One double-strand cut is produced by endonuclease *HindIII* while two cuts are made by the enzymes *HindIII*, *EcoRI* (Fig. 2c) *HpaI* and *HpaII*. On the other hand, HD-DNA is not attacked by endonuclease *BamHI*. The molecular sizes of the three HD-DNA fragments shown in Figs 2d, g are 1.90 , 0.70 , and 0.50×10^6 respectively. Treatment with restriction enzymes and gel electrophoresis were performed as before⁶.

Unlike BK, JC, polyoma, and SV40 which all share some base-sequence homology with each other^{8,9,10} HD-virus proved in DNA-DNA hybridisation studies to be unrelated to the other viruses (Table 1). Using the stringent conditions of hybridisation in $2 \times \text{SSC}$ and 50% formamide at 37°C we found, in agreement with the data of others⁸, 10–16% homology between BK and SV40, while HD and SV40 displayed only 1.2% homology. BK and HD-DNA show as little as 0.2% homology. We were unable to demonstrate any homology between HD and polyoma virus in these conditions. Furthermore, anti-SV40 T antigen and

anti-SV40 capsid sera fail to react with HD-infected VERO-cells as judged by immunofluorescence.

VERO-cells which harbour HD-virus do not display any recognisable cytopathogenic effect (c.p.e.). They are routinely passaged at a weekly 1:4 split ratio in minimum essential medium (MEM) supplemented with 10% calf serum. Because of the lack of c.p.e. we have not been able yet to establish a plaque assay for titration of the HD-virus. Thus far we have been using the synthesis of HD-superhelical FO I DNA as a measure of infectivity. Using this assay it could be shown that lysates of our VERO-cells that were obtained after freezing and thawing followed by DNase and ether treatment retained the ability to induce the synthesis of HD-FO I DNA. For this purpose 10^7 VERO-cells were frozen and thawed twice in 50 ml of MEM, the cellular debris was removed by centrifugation (10 min, 4,000 r.p.m., Christ Heraeus Minifuge), and 100 μg of pancreatic DNase (Serva) were added for 60 min at 37°C after adjusting the medium to 10 mM MgCl_2 . Then an equal volume of ether was added, the mixture was shaken for 5 min, and the medium was separated from the ether. Residual ether was removed by bubbling N_2 through the solution. Ten ml, representing one-fifth of the virus-containing solution were used after passing it through a Millipore

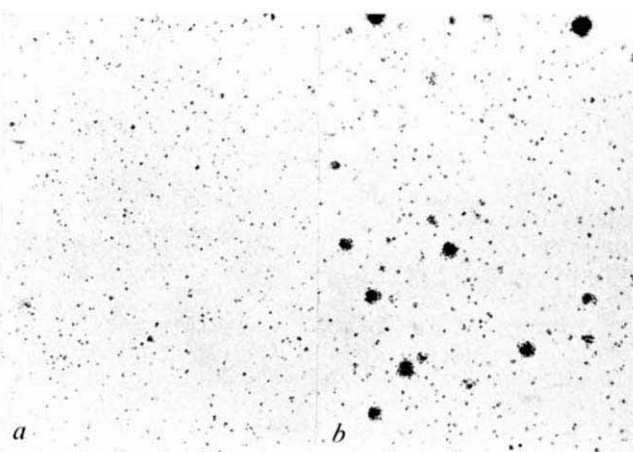


Fig. 3 Soft agar assays of a, uninfected VERO-cells (McMaster) and b, the same cells after infection with cell lysate containing HD-virus.

Millex filter (pore diameter $0.22 \mu\text{m}$) for infection of approximately 10^7 cells (1-h adsorption time). Four days later the cells were labelled with ^3H -thymidine (500 μC per 50 ml) for 1 d and the superhelical DNA was purified as before. We found that HD-DNA replicates, although poorly, in RITA cells, another permanent line derived from *Cercopithecus aethiops* (Italdiagnostics). Other cell lines such as 3T3, WI38, CV-1 and human embryonic lung proved to be non-permissive for the replication of HD-DNA.

To test whether VERO-cells that have been maintained in other laboratories also contain HD-virus or HD-viral DNA we have attempted to label such cells using the procedure described above. We were unable to demonstrate the presence of HD-FO I DNA in VERO-cells from the Institute for Virology, Universität Erlangen, and from the Department of Pathology, McMaster University, Hamilton, Ontario. VERO-cells from McMaster University totally lack HD-DNA and fail to grow in soft agar¹¹. VERO-cells grown in our laboratory contain HD-DNA and 0.1–0.5% grow to form colonies in soft agar. When the Canadian VERO-cells were infected with lysate of our VERO-cells (prepared as described above) about 1% of the cells formed colonies in soft agar (Fig. 3). The number of colonies was not increased by doubling or tripling the virus-containing inoculum. This

Table 1 DNA-DNA hybridisation between HD and other papova DNAs

³ H-DNA in solution	DNA on filter (1 μg)	³ H-c.p.m. input	³ H-c.p.m. bound	% Bound to filter*
HD	SV40	9×10^4	0	0
	BK		200	0.2
	HD		56,300	62.4
SV40	SV40	1.29×10^5	75,500	58.5
	BK		21,040	16.3
	HD		1,602	1.2
BK	SV40	6.5×10^4	6,950	10.7
	BK		49,460	76.0
	HD		0	0
Polyoma	SV40	2.2×10^4	250	1.1
	BK		0	0
	HD		0	0

Viral DNAs were labelled⁶ and purified⁶ and filter hybridisation assays⁷ were performed essentially as before.

*Corrected for nonspecific binding to T4-DNA.

might be explained by the presence of a limited number of cells, which are capable of responding to infection with the formation of colonies in soft agar. Between 0.7 and 1% of the infected cells grew to form colonies in medium containing 2% calf serum. Only 0.04% of the uninfected cells gave rise to colonies in the same conditions. This indicates a reduced serum requirement for the infected cells^{12,13}. We believe, therefore, that HD-virus can transform VERO-cells.

We have thus isolated what seems to be an entirely novel papova virus and we intend to screen human and other tumours for evidence of its distribution.

We thank K. Bosslet, T. de Ledezma, M. Pernfuss and C. Waldeck for technical assistance and J. Tooze for help in preparation of the manuscript. This work was supported by a grant of the Bundesminister für Forschung und Technologie.

Note added in proof: While this paper was in press we have found that there exists an additional size class of HD-FO 1 DNA which is by 6% larger than the HD-DNA described here.

WALDEMAR WALDECK
GERHARD SAUER

Institut für Virusforschung,
Deutsches Krebsforschungszentrum,
6900 Heidelberg, Germany

Received 11 March; accepted 12 July 1977.

- ¹ Gardner, S. D., Field, A. M., Coleman, D. V. & Hulme, B. *Lancet* **i**, 1253 (1971).
- ² Padgett, P. L., Walter, D. L., Zu Rhein, G. M., Eckroade, R. J. & Dessel, B. H. *Lancet* **i**, 1257 (1971).
- ³ Gardner, S. D. *Br. med. J.* **1**, 77 (1973).
- ⁴ Yasumura, T. & Kawakita, Y. *Nippon Rinsho* **21**, 1201 (1963).
- ⁵ Radloff, R., Bauer, W. & Vinograd, J. *Proc. natn. Acad. Sci. U.S.A.* **57**, 1514 (1967).
- ⁶ Waldeck, W., Chowdhury, K., Gruss, P. & Sauer, G. *Biochim. biophys. Acta* **425**, 157 (1976).
- ⁷ Kuhn, C., Waldeck, W. & Sauer, G. *Z. Krebsforsch.* **83**, 65 (1975).
- ⁸ Khoury, G., Howley, P. M., Baron, G. & Mullerkey, M. F., *Proc. natn. Acad. Sci. U.S.A.* **72**, 2563 (1975).
- ⁹ Osborn, J. E., Robertson, S. M., Padgett, B. L., Walter, D. L. & Weisblum, B. *J. Virol.* **19**, 675 (1976).
- ¹⁰ Ferguson, J. & Davis, R. W. *J. molec. Biol.* **94**, 496 (1976).
- ¹¹ Macpherson, I. & Montagnier, L. *Virology* **23**, 291 (1964).
- ¹² Clarke, G. D., Stoker, M. G. P., Ludlow, A. & Thornton, M. *Nature* **227**, 798 (1970).
- ¹³ Dulbecco, R. *Nature* **227**, 802 (1970).

Type C RNA virus production and cell competence for normal differentiation in myeloid leukaemic cells

STUDIES with various species have shown that type C viral genomes can be integrated and vertically transmitted as chromosomal elements within the cellular genomes (for review see refs 1–6) and that nucleic acid sequences derived from these viruses can be detected in the DNA^{7–10} of normal cells (for review see ref. 11). It has been suggested that in addition to their role in tumour formation, type C RNA viruses may also have a role in normal cell differentiation^{2,3}. We suggest here that one way in which these viruses may influence differentiation, is by modifying the competence of cells to be induced to differentiate by a normal regulator.

Normal differentiation-inducing regulators have been identified in cells of the haematopoietic system and these include the protein erythropoietin¹² which induces differentiation of erythrocytes and the protein that we now call MGI (macrophages and granulocyte inducer) (refs 13, 14) which induces differentiation of macrophages and granulocytes^{13,15–18}. The isolation of clones of haematopoietic cells with different degrees of competence for differentiation by one of these proteins, should be a useful experimental system to test the relationship between type C RNA viruses and cell competence for differentiation by a specific regulator. We have isolated such clones from

mouse myeloid leukaemia, which differ in their competence to be induced to differentiate by MGI (refs 13, 19–23). One type of clone, MGI⁺D⁺, can be induced by MGI to undergo normal cell differentiation, including the formation of rosettes for cell surface Fc and C3 receptors^{20,21}, the synthesis and secretion of lysozyme²² and differentiation to mature macrophages and granulocytes^{13,19}. A second type of clone, MGI⁺D⁻, can be induced for Fc and C3 rosettes and lysozyme but not to differentiate to mature cells and a third type, MGI⁻D⁻, was not inducible for any of these properties (Table 1). Some, but not all the properties associated with cell differentiation can also be induced in some of these myeloid leukaemic clones by the steroid hormones dexamethasone, prednisolone or oestradiol^{20,21,24} and compounds such as cytosine arabinoside, actinomycin D, 5-iododeoxyuridine²⁰ or dimethylsulphoxide²². The protein inducer^{13–18} that we call MGI has also been referred to by others as colony stimulating factor²³ or activity²⁶. We show here that the competence of myeloid leukaemic clones to be induced to differentiate to mature macrophages and granulocytes by the normal regulator MGI, is associated with a higher production of type C virus.

Table 1 Competence for the induction of differentiation in clones of myeloid leukaemic cells by the differentiation-inducing protein MGI

Cell type	Clone numbers	Inducibility by MGI			
		Rosettes		Mature macrophages and granulocytes	
		Fc	C3	Lysozyme	
MGI ⁺ D ⁺	9,11,12	+	+	+	+
MGI ⁺ D ⁻	5,13,19	+	+	+	—
MGI ⁻ D ⁻	1,6,10	—	—	—	—

(+), Inducible, (—), non-inducible. Cells were incubated with conditioned medium containing MGI as described²³. MGI⁺D⁺ clones 9,11,12 and MGI⁺D⁻ clones 5,13,19 (refs 20, 21) were derived from a myeloid leukaemia originating in a SL mouse²⁶. MGI⁻D⁻ clones 1,6,10 (refs 21–23) were derived from three independently induced myeloid leukaemias after X-ray irradiation of SJL/J mice. Before treatment with MGI, cells from all the clones grew in suspension as myeloblasts to promyelocytes and produced myeloid leukaemia after inoculation into isologous adult mice. The cells were cultured in Eagle's medium with a fourfold concentration of amino acids and vitamins (H-21, GIBCO) and 10% inactivated (56 °C, 30 min) foetal calf serum.

All the nine clones tested of the three types shown in Table 1 were found to be producing type C virus. Typical type C particles were observed by electron microscopy in sections of the cultured cells and at a buoyant density of 1.15gcm⁻³ in a sucrose equilibrium density gradient of pellets from the culture medium. To quantitate the amount of virus released by the different clones, we measured the reverse transcriptase activity⁴ of partially purified and concentrated virus pellets obtained from the culture fluids²⁸. The virus production measured by this enzyme activity with the template oligo(dT)-poly(rA), and cellular growth rate of three clones representative of the different types of myeloid leukaemic cells are shown in Fig. 1. The MGI⁺D⁺ and the MGI⁺D⁻ clones had similar growth rates, while the MGI⁻D⁻ clones grew somewhat faster. Virus production in all the clones was not significantly different for the first 2 d after seeding. When the cells entered the logarithmic phase of growth, however, virus production increased 16-fold in the MGI⁺D⁺ clone and only 2-fold in the MGI⁺D⁻ and MGI⁻D⁻ clones. Optimum virus production was obtained with all three types of clones at day 4 and then declined when the cells approached the stationary phase of growth. A similar relationship between type C RNA virus production and cellular growth has been observed in cultures of

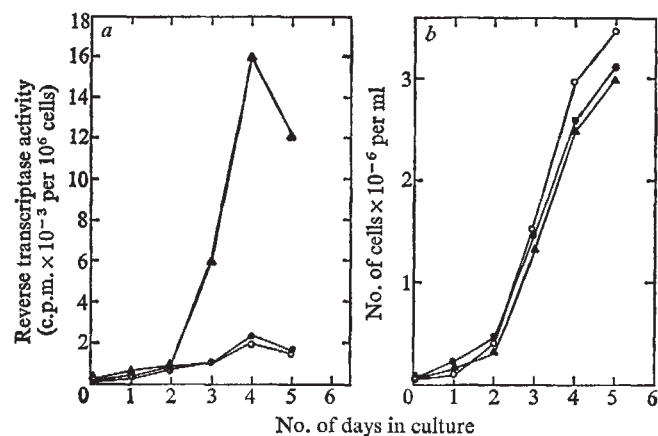


Fig. 1 Reverse transcriptase activity in the tissue culture medium (a) and number of cells (b) at different days after seeding of the three types of myeloid leukaemic clones. ▲, MGI⁺D⁺ clone 9 and ●, MGI⁺D⁻ clone 5 were seeded at 4×10^5 cells and ○, MGI⁻D⁻ clone 6 at 2×10^5 cells per 10-cm Nunc tissue culture Petri dish in 10 ml modified Eagle's medium (see Table 1) and 10% inactivated foetal calf serum. To assay for reverse transcriptase activity⁴, the cells were pelleted from 20 ml of medium and the medium clarified of cell debris²⁸ by centrifugation at 10,000 r.p.m. in a SS-34 rotor in a RC-5 Sorval centrifuge at 4 °C. The clarified medium was layered on a 5-ml cushion of 20% (v/v) glycerol in TNE buffer (0.1 M NaCl, 0.01 M Tris pH 7.6, and 0.001 M EDTA) and centrifuged at 40,000 r.p.m. for 60 min in a 60TI type rotor at 4 °C in a Beckman ultracentrifuge. The resultant pellets were suspended in 0.1 ml TNE buffer. Enzyme activity was measured using [the synthetic template oligo(dT)-poly(rA) (Collaborative Research). Oligo(dT) and poly(rA) were pre-annealed at a ratio of 1:10 in 0.15 M NaCl, 10 mM Tris-hydrochloride (pH 7.8) at 37 °C for 5 min. Enzyme reaction mixtures (50 μ l) consisted of 2 μ M Tris (pH 7.8) 6 μ M NaCl, 0.1 μ M MnCl₂, 0.4 μ M DTT, 0.015% NP/40, 5 μ g oligo(dT)-poly(rA), 0.001 μ M unlabelled TTP, 2 μ Ci H³-dTTP, and 10 μ l of the suspended virus pellet in TNE buffer. Reactions were carried out for 30 min at 37 °C and terminated by addition of 0.1 ml 0.2 M sodium pyrophosphate and 1 ml 10% trichloroacetic acid. Samples were collected on 0.45 μ m Millipore filters rinsed with 5% cold trichloroacetic acid and counted in a toluene-based scintillation fluid in a Tri-carb Packard fluid scintillation counter. The values represent the means of three independent experiments.

other cell types²⁹⁻³². Since virus production in our experiments reached an optimum at day 4, all the following experiments were carried out on the fourth day after seeding.

Studies with the 9 clones have shown (Fig. 2), that only the three MGI⁺D⁺ clones, which can be induced to differentiate to mature macrophages and granulocytes, produced high levels of virus, while the partially inducible MGI⁺D⁻ and the non-inducible MGI⁻D⁻ clones produced lower levels of virus. The amount of virus produced by the MGI⁺D⁺ clones was about 4-5-fold higher than the highest amount produced by any of the MGI⁺D⁻ or MGI⁻D⁻ clones (Fig. 2). Three subclones isolated from MGI⁺D⁺ clone 9 and three subclones from MGI⁺D⁻ clone 5 were assayed for reverse transcriptase activity. All the MGI⁺D⁺ subclones showed the same high enzyme activity in the culture medium as the parental clone 9, while all the MGI⁺D⁻ subclones showed the lower enzyme activity of the parental clone 5.

The higher enzyme activity in the culture fluids from MGI⁺D⁺ clones was further identified as type C virus reverse transcriptase, by sucrose equilibrium density gradient centrifugation of the partially purified virus preparations from the three types of myeloid leukaemic clones. The peak of the reverse transcriptase activity at the buoyant density of 1.15 g cm^{-3} was 10-fold higher with MGI⁺D⁺ clone 9 than with MGI⁺D⁻ clone 5 and MGI⁻D⁻ clone 6. The virus specific nature of the enzyme was also confirmed by using, in addition to oligo(dT)-poly-

(rA), two other synthetic templates, oligo(dG) poly(rC), a viral specific template, and oligo(dT) poly(dA), which is utilised poorly by the viral RNA directed DNA polymerase and is a good template for the cellular DNA-DNA polymerases³³⁻³⁶ (for review see ref. 37). The results have shown that with pellets obtained from culture fluids of all the 9 clones, the highest activity with oligo(dG) poly(rC) was again found with the MGI⁺D⁺ clones and there was almost no activity with oligo(dT) poly(dA). Incubation of cells for 1 d with 50 units of purified mouse interferon (from Dr I. Gresser, Institut de Recherches Scientifiques sur le Cancer, Villejuif), gave 70%-80% reduction in the enzymatic activity that could be recovered from the culture medium. This is in agreement with previous results showing that interferon can inhibit the secretion of type C virus³⁸⁻⁴⁰, and provides additional evidence for the viral nature of the activity measured.

Indirect immunofluorescence tests⁴¹ with an antiserum against murine leukaemia virus group-specific (MULV-gs) antigens (from Dr M. Haas⁴²) showed that all three types of clones were positive for these viral antigens and that all the cells were stained. After acetone fixation, MGI⁺D⁺ clones seemed to be the most brightly stained and titration of MULV-gs antigens by absorption of immunofluorescence with cell extracts⁴² has indicated, that the MGI⁺D⁺ clones had a higher amount of MULV-gs antigens. Unfixed cells tested for cap formation also showed a higher frequency of caps for MULV-gs antigens on MGI⁺D⁺ cells (Table 2).

Table 2 MULV-gs antigens. Titre of cell extracts tested by immunofluorescence absorption, and cap formation on unfixed cells

Cell type	Clone no.	Immunofluorescence absorption, antigen dilution of cell extracts						% Cells with cap
		0	1:2	1:4	1:8	1:16	1:32	
MGI ⁺ D ⁺	9	—	—	—	—	++	+++	90 $\pm$ 5
MGI ⁺ D ⁻	5	—	—	+	++	+++	+++	10 $\pm$ 3
MGI ⁻ D ⁻	6	—	+	++	+++	+++	+++	2 $\pm$ 1

MULV-gs antigen titre of cell extracts was tested by absorption of immunofluorescence. Cells at 4 d after seeding were sonicated in Earl's balanced salt solution to give a 20% cell extract. Serial dilutions of this extract (40 μ l) were then incubated overnight with an equal volume of Lewis rat anti-MULV-gs antiserum⁴² at 4 °C. Residual immunofluorescence after incubation with fluorescent rabbit anti-rat IgG (Miles Yeda) was scored on an acetone fixed NIH/3T3 Moloney virus producing mouse cell line on a scale from — (no immunofluorescence) to +++ (strong immunofluorescence obtained without absorption). Absorption tests were performed with a concentration of antiserum (1:32) two doubling dilutions below the end-point for the acetone fixed NIH/3T3 Moloney virus producing mouse cells. Cap formation on cells 4 d after seeding was tested after incubation of unfixed cells with rat anti-MULV-gs antiserum (1:8) for 15 min and then for 30 min with fluorescent rabbit anti-rat IgG. There was no immunofluorescence after incubation with normal rat serum instead of the anti-MULV-gs antiserum.

Our results show that mouse myeloid leukaemic cells that can be induced to differentiate to mature macrophages and granulocytes by the normal inducer protein MGI, produced a higher amount of type C RNA virus than clones of myeloid leukaemic cells that can be only partially or not at all induced to differentiate by this normal regulator. The amount of virus production was, therefore, associated with the competence of the leukaemic cells to undergo differentiation to normal mature cells. It will be of interest to identify the virus or viruses produced. Type C RNA viruses have been observed in myeloid leukaemic cells from human and rats⁴³⁻⁴⁵, and it will also be of interest to determine in these two other species, the relationship between the amount of virus production and ability of the cells to be induced to differentiate by the normal regulator MGI.

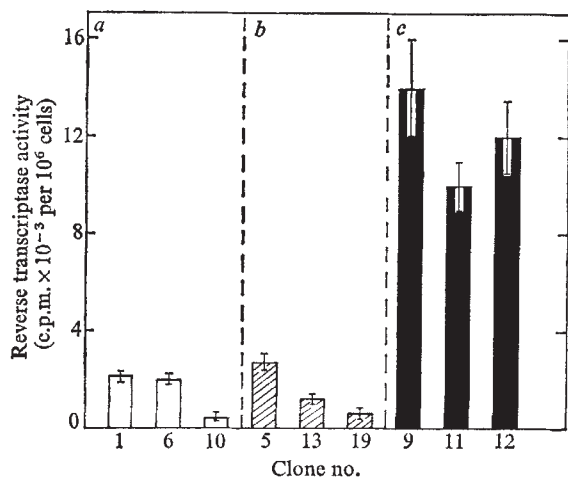


Fig. 2 Reverse transcriptase activity in the culture fluids of nine myeloid leukaemic clones. MGI⁺D⁺ clones 9,11,12 (c) and MGI⁺D⁻ clones 5,13,19 (b) were seeded at 4×10^5 cells and MGI⁻D⁻ clones 1,6,10 at 2×10^5 cells (a) per 10-cm tissue culture Petri dish. The culture medium was collected 4 d after seeding and reverse transcriptase activity measured as described in the legend to Fig. 1. The means and standard errors are based on six independent experiments.

Friend erythroleukaemic cells can be induced to differentiate partially by dimethylsulphoxide⁴⁶ and some other polar compounds⁴⁷ and the competence for this partial induction of erythroid differentiation was not associated with the amount of virus production³². However, the erythroleukaemic cells studied had lost the ability to respond to erythropoietin⁴⁸, the normal regulator of erythroid cell differentiation, whereas the MGI⁺ myeloid leukaemic cells, including those that can respond to dimethylsulphoxide²², have not lost the ability to respond to MGI, the normal regulator of differentiation to macrophages and granulocytes. It may also be that the viruses produced by the Friend erythroleukaemic cells differ from those produced by the myeloid leukaemic cells and that not all type C viruses are related to cell competence for differentiation.

One possible explanation for the relationship that we have found in our myeloid leukaemic cell system may be the association of viral sequences with regulatory sites for differentiation. The increased virus production could then indicate a different state of the regulatory sites in the competent compared with the non-competent cells. The higher virus production by the competent cells could either be a by-product or directly influence these regulatory sites. Another possibility is that the increased virus production by the competent cells is associated with an appropriate arrangement of surface receptors for MGI that is required for the induction of normal cell differentiation. The differences in cap formation by MULV-antigens (Table 2) and con A (refs 13, 49) indicate that it will be of interest to determine whether, as in cells transformed by sarcoma viruses⁵⁰, there are also differences in receptors for other polypeptide hormones. The myeloid leukaemic clones used in our studies seem to be a particularly favourable experimental system to investigate further these possibilities of the role of type C viruses in relation to cell competence for differentiation by a normal receptor.

We thank Dr M. Haas for help in experiments using anti-MULV-gs antiserum and Mrs Haya Dorf for technical assistance.

DAN LIEBERMANN
LEO SACHS

Department of Genetics,
Weizmann Institute of Science,
Rehovot, Israel

Received 18 May; accepted 11 July 1977.

- Gross, L. *Oncogenic Viruses* (Pergamon, New York, 1970).
- Huebner, R. J. & Tadaro, G. J. *Proc. natn. Acad. Sci. U.S.A.* **64**, 1087-1094 (1969).
- Temin, H. M. J. *natn. Cancer Inst.* **46**, III-VII (1971).
- Temin, H. M. & Baltimore, D. *Adv. Virus Res.* **17**, 129-186 (1972).
- Toozé, J. *The Molecular Biology of Tumour Viruses* (Cold Spring Harbor Laboratory, New York, 1973).
- Gillespie, D., Saxinger, W. C. & Gallo, R. C. *Prog. Nucleic Acid Res. molec. Biol.* **15**, 1-108 (1975).
- Gelb, L. D., Aaronson, S. A. & Martin, M. A. *Science* **172**, 1353-1355 (1971).
- Baluda, M. A. *Proc. natn. Acad. Sci. U.S.A.* **69**, 576-580 (1972).
- Neiman, P. E. *Virology* **53**, 196-203 (1973).
- Gelb, L. D., Milstein, J. B., Martin, M. A. & Aaronson, S. A. *Nature new Biol.* **244**, 76-79 (1973).
- Aaronson, S. A. & Stephenson, J. R. *Biochim. biophys. Acta* **458**, 323-354 (1976).
- Krantz, S. B. & Jacobson, L. O. *Erythropoietin and the Regulation of Erythropoiesis* (University of Chicago Press, Chicago, 1970).
- Sachs, L. in *Harvey Lectures* **68**, 1-35 (Academic, New York, 1974).
- Landau, T. & Sachs, L. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2540-2544 (1971).
- Pluznik, D. H. & Sachs, L. *J. cell. comp. Physiol.* **66**, 319-324 (1965).
- Pluznik, D. H. & Sachs, L. *Expl Cell Res.* **43**, 553-563 (1966).
- Ichikawa, Y., Pluznik, D. H. & Sachs, L. *Proc. natn. Acad. Sci. U.S.A.* **56**, 488-495 (1966).
- Bradley, T. R. & Metcalf, D. *Aust. J. exp. Biol. med. Sci.* **44**, 287-299 (1966).
- Fibach, E., Hayashi, M. & Sachs, L. *Proc. natn. Acad. Sci. U.S.A.* **70**, 343-346 (1973).
- Lotem, J. & Sachs, L. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3507-3511 (1974).
- Lotem, J. & Sachs, L. *J. Immun.* **117**, 580-586 (1976).
- Krystosek, A. & Sachs, L. *Cell* **9**, 675-684 (1976).
- Azumi, J. & Sachs, L. *Proc. natn. Acad. Sci. U.S.A.* **74**, 253-257 (1977).
- Lotem, J. & Sachs, L. *Int. J. Cancer* **15**, 731-740 (1975).
- Metcalf, D. *J. cell Physiol.* **74**, 323-332 (1969).
- Austin, P. E., McCulloch, E. A. & Till, J. E. *J. cell Physiol.* **77**, 121-134 (1971).
- Ichikawa, Y. *J. cell Physiol.* **74**, 223-234 (1969).
- Lieber, M. M., Livingstone, P. M. & Todaro, G. F. *Science* **181**, 443-444 (1974).
- Humphries, E. H. & Temin, H. M. *J. Virol.* **14**, 531-546 (1974).
- Greenberger, J. S. & Aaronson, S. A. *J. Virol.* **15**, 64-70 (1975).
- Fischinger, P. J., Tuttle-Fuller, N., Huper, G. & Bolognesi, D. P. *J. Virol.* **16**, 267-274 (1975).
- Sherton, C. C., Evans, L. H., Polonoff, E. & Kabat, D. J. *J. Virol.* **19**, 118-125 (1976).
- Weissbach, A., Bolden, A., Muller, R., Hanafusa, H. & Hanafusa, T. J. *J. Virol.* **10**, 321-327 (1972).
- Baltimore, D. & Smoler, D. *Proc. natn. Acad. Sci. U.S.A.* **68**, 1507-1511 (1970).
- Spiegelman, S. *et al. Nature* **228**, 430-432 (1970).
- Goodman, N. C. & Spiegelman, S. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2203-2206 (1971).
- Sarnagadharan, M. G., Alloudeen, H. S. & Gallo, R. C. *Meth. Cancer Res.* **12**, 3-47 (1976).
- Liebermann, D., Voloch, Z., Aviv, H., Nudel, U. & Revel, M. *Molec. Biol. Rep.* **1**, 447-451 (1974).
- Sweetly, P. & Ostertag, W. *Nature* **251**, 642-644 (1974).
- Metz, D. H. *Cell* **6**, 429-439 (1975).
- Hilgers, J., Nowinski, R. C., Geering, G. & Hardy, W. *Cancer Res.* **32**, 98-106 (1972).
- Haas, M. J. *natn. Cancer Inst.* **58**, 251-257 (1977).
- Mak, T. W., Manaster, J., Howatson, A. F., McCulloch, E. A. & Till, J. E. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4336-4340 (1974).
- Gallagher, R. E., Salahuddin, S. Z., Hall, W. T., McCredie, K. B. & Gallo, R. C. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4137-4141 (1975).
- Greenberger, J. S., Aaronson, S. A., Rosenthal, D. S. & Moloney, W. C. *Nature* **257**, 143-144 (1975).
- Friend, C., Scher, W., Holland, J. G. & Sato, T. *Proc. natn. Acad. Sci. U.S.A.* **68**, 378-382 (1971).
- Reuben, R. C., Wife, R. L., Breslow, R., Rifkind, R. & Marks, P. A. *Proc. natn. Acad. Sci. U.S.A.* **73**, 862-866 (1976).
- Kluge, N., Gadicke, G., Steinheider, G., Dube, S. & Ostertag, W. *Expl Cell Res.* **88**, 257-262 (1974).
- Lotem, J., Vlodavsky, I. & Sachs, L. *Expl Cell Res.* **101**, 323-330 (1976).
- Todaro, G. J., DeLarco, J. E. & Cohen, S. *Nature* **264**, 26-31 (1975).

Post-transcriptional control of avian oncornavirus transforming gene sequences in mammalian cells

TRANSFORMATION of cells by RNA tumour viruses generally results in a stable association of the viral genome with the morphologically altered cell. Studies with temperature-sensitive mutants of these viruses have clearly demonstrated that viral-specific gene functions are required for establishment and maintenance of the transformed phenotype¹⁻⁵. For avian oncornaviruses the transforming (sarcoma) gene sequences are required for both transformation of cells in tissue culture and the production of tumours in animals⁷⁻⁸. In all of the oncornavirus-infected mammalian cell systems studied to date regulation of expression of the avian RNA tumour virus transforming gene sequences seems to be under the influence of transcriptional control mechanism(s) because appreciable differences in the amount of sarcoma-specific RNA can be detected in cells exhibiting normal and transformed phenotypes. For instance, the amount of sarcoma-specific RNA was substantially reduced in most revertant subclones of Rous sarcoma virus (RSV)-infected hamster cells compared with the original transformed clones⁹⁻¹¹. Although several revertant RSV-hamster cells exhibited less dramatic differences in sarcoma-specific RNA, it nevertheless seemed that the transformed

phenotype was directly related to the extent of transcription of the virus transforming gene sequences in this transformed/revertant cell system. A similar correlation between the amount of sarcoma-specific viral RNA and the malignant phenotype was recently reported for murine sarcoma virus-infected cells¹².

Here we present data indicating that mammalian cells may also regulate the expression of viral transforming gene sequences by post-transcriptional mechanisms. This conclusion is based on the observation that revertant subclones of RSV-infected field vole cells contain as much, if not more, sarcoma-specific RNA as the transformed cells from which they were derived. Our preliminary data also indicate that the post-transcriptional restriction of expression of viral transforming genes in revertant vole cells does not result from either selective transport of sarcoma-specific RNA from the nucleus to the cytoplasm, or the lack of association of this RNA species with polyribosomes.

An established diploid cell line from the European field vole, *Microtus agrestis* was transformed by the Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV) by Dr P. Vogt of the University of Southern California. The procedures for the isolation of clones of RSV-transformed mammalian cells and revertant subclones have been described elsewhere^{13,14}. The morphology of typical normal, transformed, and revertant vole cell types can be seen in Fig. 1. Not only did the revertant subclones resemble normal cells in their 'flat' fibroepithelial-like morphology but they also failed to grow in soft agar (R. Krzyzek, unpublished observations).

We have previously demonstrated that both transformed and revertant field vole cells contain similar genome-equivalents of virus-specific DNA¹⁵. These studies were performed by determining the influence, if any, that unlabelled DNA from RSV-transformed, and reverted vole cells has on the reassociation kinetics of the more complex double-stranded DNA component synthesised by the RSV RNA-directed DNA polymerase *in vitro*¹⁶. However, these methods cannot completely exclude the possibility that a small portion (<20%) of the viral genome is deleted from the DNA of the reverted cell because the virus-specific DNA probe used in these studies may not represent the entire viral genome¹⁸. Consequently it was conceivable that the reversion phenomenon could result from the complete or partial deletion of the RSV-transforming gene sequences. To determine whether the RSV-transforming gene sequences were retained in vole cells during reversion we have

performed further hybridisation analysis with a specific radiolabelled DNA probe complementary to the RSV-transforming gene sequences (cDNA_{src})¹⁷. As shown in Table 1 the cDNA_{src} probe hybridises equally well with DNA obtained from either transformed or revertant field vole cells. The extent of hybridisation is not complete in our conditions of hybridisation even with DNA from RSV-transformed avian cells. The reason for this is not clear; however this problem is not unique to our situation but has been observed by other investigators as well¹. Even if complete hybridisation was achieved, this would not negate the possibility that the transforming gene in the revertant cell type was inactive due to a very small gene deletion or a point mutation which would not be detectable by the hybridisation techniques used. Therefore we performed studies to determine whether the transforming gene sequences present in the revertant vole cells were biologically active and capable of inducing transformation. As indicated in Table 2, the entire infectious RSV(D) genome could be rescued from transformed and revertant cells by either fusion with permissive cells or transfection of avian cells with DNA purified from both cell types. Although we have not computed precise frequencies of rescue, the efficiency of rescue of virus from transformed and revertant cells by both techniques was quite similar.

Table 1 Hybridisation of cDNA_{src} to transformed and revertant field vole cell DNA

Source of cellular DNA	Hybridisation (%)
RSV-infected duck	49.5
RSV-transformed field vole	
Clone 1	43.0
Clone 22	33.0
Revertant field vole	
Subclone 866	42.0
Subclone 4	44.6
Field vole liver	0.0

Cellular DNA was prepared as previously described¹⁹ and fragmented to a length of 300–400 nucleotides by treatment with 0.3 N NaOH for 30 min at 100 °C. Hybridisation analysis was performed in 30- μ l reaction volumes containing 0.6 M NaCl, 0.02 M Tris-HCl (pH 7.4) 0.002 M EDTA, 0.05% SDS, ³H-cDNA_{src} (1,500 c.p.m.) and a vast excess of fragmented, denatured cellular DNA (12 mg ml⁻¹) to *C₀t* values of approximately 5×10^4 mol l⁻¹. All reactions were incubated for 70 h at 68 °C and the extent of hybridisation was determined by resistance to S₁ nuclease^{20,21}. % hybridisation was corrected for the intrinsic S₁ nuclease-resistance of cDNA_{src} (13.0%).

Since the revertant vole cells contain the entire biologically active sarcoma virus genome, it was of interest to determine whether an alteration in the transcriptional expression of the proviral DNA genome was responsible for phenotypic reversion. Our previous studies had indicated that revertant field vole cells contained similar amounts of virus-specific RNA as the transformed cell types (R. Krzyzek, A.F.L., P. Vogt & A.J.F., submitted for publication). The cDNA probe used in these studies was a relatively uniform representation of the viral RNA genome and indicated that the bulk (~70%) of the RSV-specific nucleotide sequences were expressed as virus-specific RNA in both transformed and revertant cells. However, this probe was not sufficiently sensitive to determine whether differences in transcription of the viral transforming gene occurred in these cell types. Therefore, to determine directly the expression of the RSV-transforming gene sequences in transformed and revertant vole cells we used the ³H-labelled cDNA_{src} probe to quantitate sarcoma-specific RNA in these cells. From Fig. 2 it can be seen that the rates of hybridisation of the cDNA_{src} probe to total cell RNA obtained from both transformed and revertant field vole cells was not appreciably different,

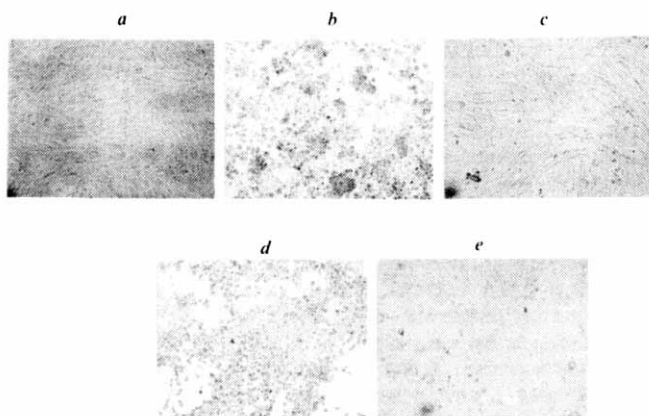


Fig. 1 Morphology of RSV-transformed and revertant field vole cells. *a*, Normal field vole cells; *b*, RSV-transformed field vole cells (clone 1); *c*, Revertant field vole cells (subclone 866); *d*, RSV-transformed field vole cells (clone 22); *e*, Revertant field vole cells (subclone 4). All photographs were taken at 80 $\times$ magnification.

Table 2 Rescue of Rous sarcoma virus from transformed and revertant field vole cells by DNA transfection and Sendai virus-mediated fusion with susceptible chicken cells

Cell type	Transfection	Sendai virus-mediated fusion
RSV-transformed field cells		
Clone 1	+	+
Clone 22	ND	+
Revertant field vole cells		
Subclone 4	ND	+
Subclone 866	+	+
Uninfected field vole cells	—	—

Cellular DNA was prepared from field vole cells by the method of Haase *et al.*²². Transfections were performed by coprecipitation of DNA with calcium phosphate^{23,24}. Briefly, DNA dissolved in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate (HEPES)-saline-phosphate buffer at 20 $\mu\text{g ml}^{-1}$ was adjusted to 0.125 M CaCl_2 and allowed to precipitate at room temperature for 30 min. Aliquots (0.5 ml) of the DNA-calcium phosphate mixture containing approximately 10 μg of DNA were then added to chick secondary fibroblasts ($0.5\text{--}10^6$ cells per 60-mm tissue culture plate) previously washed twice with (HEPES)-saline-phosphate buffer. After 30 min incubation at room temperature, 2.5 ml of growth medium containing 10% calf serum but without tryptose phosphate broth was added to the cultures which were then incubated for 4 h at 37 °C. The growth medium containing the DNA-calcium phosphate mixture was then removed and replaced with growth medium containing tryptose-phosphate broth with 10% calf serum. Foci of transformed cells appeared within 10 to 14 d. When revertant or RSV-transformed field vole cell DNA was treated with pancreatic DNase (10 $\mu\text{g ml}^{-1}$, 30 min at room temperature) before the addition of CaCl_2 , no transformation of chick fibroblasts was observed.

Rescue of RSV from field vole cells by Sendai-mediated fusion with chick fibroblasts was performed as described by Boettiger²⁵. The procedure involved adding 0.1 ml of β propiolactone-inactivated Sendai virus (200–400 haemagglutination units) to cultures of chick secondary fibroblasts ($0.5 \times 10^6\text{--}1.0 \times 10^6$ cells per 35-mm tissue culture plate) previously washed twice with cold growth medium without serum. The cultures were then incubated for 15 min at 4 °C, with cold growth medium without serum and 0.1 ml containing 5×10^5 field vole cells previously treated with mitomycin C (10 $\mu\text{g ml}^{-1}$, 2 h at 37 °C) was added. After incubation for 40 min at 37 °C, growth medium was added containing 10% calf serum. Foci of transformed cells appeared within 4–7 d after fusion. No transformation was observed if treatment of chick fibroblasts with Sendai virus was omitted. Coincident with the appearance of foci was the production of virus as assayed by particle count and focus formation. Identification of the virus as RSV subgroup D was accomplished serologically by demonstrating that infectivity of the rescued virus was neutralised by RSV-D-specific antisera.

ND, Not done.

indicating the presence of similar amounts of sarcoma-specific RNA in both cell types. In fact revertant subclone 866R actually contained more sarcoma-specific RNA than both the transformed clones. Furthermore, the maximum hybridisation achieved from both cell types was identical, indicating that similar extents of transcription of the sarcoma-specific sequences had occurred in transformed and revertant cells. The extent of hybridisation with RNA from transformed and revertant field vole cells was identical to that observed with both RNA from transformed permissive cells and purified 70S RNA from the same strain of RSV used to synthesise the cDNA_{src} probe. No hybridisation to the cDNA_{src} probe was observed with RNA from normal field vole cells (Fig. 2).

In an effort to determine whether the sarcoma-specific RNA sequences were distributed similarly in transformed and revertant field vole cells, nuclear, cytoplasmic and polyribosomal RNA from these cells were hybridised to the cDNA_{src} probe (Figs 3 and 4). The rates of hybridisation obtained with the nuclear and cytoplasmic fractions indicated that the amounts of sarcoma-specific RNA present in the revertant cells were no less than that found in the corresponding fractions of the transformed cells. Thus it seems unlikely that the reverted state is related to an alteration in the intracellular localisation of sarcoma-specific RNA in these cells. Similar results were obtained for RNA isolated from polyribosomes

from both cell types indicating that the same amount of sarcoma-specific RNA is associated with polyribosomes in the revertant and transformed field vole cells (Fig. 4).

The hybridisation analyses presented above between RNA from transformed and revertant vole cells and a cDNA probe representing sarcoma-specific RNA sequences indicate that the phenomenon of reversion in these cells is not a function of transcriptional regulation of the

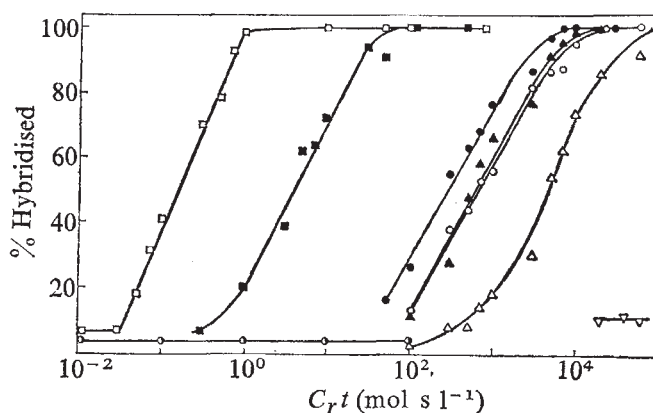


Fig. 2 Detection and quantitation of sarcoma-specific RNA[†] in transformed and revertant field vole cells. Total cellular RNA was extracted from vole cells with sodium dodecyl sulphate-phenol at 56 °C as previously described²⁶. Nucleic acids were precipitated with ethanol and collected by centrifugation at 113,000g for 30 min. The pellets were dissolved in DNase buffer (0.01 M Tris-HCl (pH 7.4), 0.01 M MgCl_2 at a concentration of 0.5 to 1.0 mg ml^{-1} . Residual DNA was removed by digestion with 20 μg of RNase-free pancreatic DNase ml^{-1} for 2 h at room temperature²⁷. The preparations were then adjusted to 0.01 M EDTA, 0.1 M Tris-HCl (pH 9.0), 0.5% SDS, extracted three times with two volumes of phenol-chloroform-isoamyl alcohol (50:50:1), precipitated twice with ethanol, and resuspended in TE buffer (0.02 M Tris-HCl, pH 7.4, 0.01 M EDTA). The conditions for the synthesis of virus-specific DNA *in vitro* with detergent-activated RSV are essentially those described by Stehelin *et al.*¹⁷. Reaction mixtures contained 200 $\mu\text{g ml}^{-1}$ of virus protein; 0.1 M Tris-HCl (pH 8.1); actinomycin D (100 $\mu\text{g ml}^{-1}$); 0.008 M MgCl_2 ; 2% (v/v) β -mercaptoethanol; unlabelled dATP, dCTP and dGTP (each 2×10^{-4} M); ^3H -dTTP (6.6×10^{-6} M, 0.33 mCi ml^{-1}); and Nonidet P-40 (0.08% v/v). After incubation for 4 h at 37 °C, the reaction mixture was supplemented with the addition of unlabelled dATP, dCTP, and dGTP to 4×10^{-4} M, further incubated for 12 h and then terminated by the addition of EDTA to 0.01 M. The enzymatic product was extracted with SDS-Pronase-phenol, treated with 100 $\mu\text{g ml}^{-1}$ pancreatic RNase for 1 h at 37 °C in 0.003 M EDTA to disrupt RNA:DNA hybrids, re-extracted with SDS-Pronase-phenol and concentrated by ethanol precipitation. cDNA complementary to the RSV transforming gene sequences (cDNA_{src}) was then purified from the total viral-specific DNA product by selective hybridisation to avian leukosis virus essentially as described by Stehelin *et al.*¹⁷. The transformation specific nature of this probe was confirmed by demonstrating that cDNA_{src} hybridises to transforming sarcoma virus RNA but not to non-transforming leukosis virus RNA. Hybridisation of ^3H -labelled cDNA_{src} to vole cell RNA was performed in a 10- μl reaction mixture containing 0.01 M Tris-HCl (pH 7.4), 0.6 M NaCl, 0.001 M EDTA, single-strand ^3H -cDNA (1,500 c.p.m.), 1.0 mg ml^{-1} yeast tRNA and the indicated amount of RNA. The reaction mixtures were sealed in 30- μl microcaps and incubated for either 24 or 70 h at 68 °C. Hybrid formation was detected by treatment of samples with single-strand specific S_1 nuclease^{20,21}. The results of hybridisation analysis were expressed as a function of C_t (concentration of RNA in $\text{mol l}^{-1} \times$ time of incubation in s). The percent hybridisation was corrected for the intrinsic S_1 nuclease resistance of cDNA_{src} (13%) and normalised to 100% values (actual hybridisation averaged 70–75%). Each point represents the average of three separate determinations. $\square$, RNA 70S RNA; $\blacksquare$, RSV-infected duck cells; $\circ$, RSV-transformed field vole clone 1; $\triangle$, RSV-transformed field vole clone 22; $\blacktriangle$, revertant field vole subclone 4; $\bullet$, revertant field vole subclone 866; Δ , uninfected field vole; $\ominus$, avian leukosis virus 70S RNA.

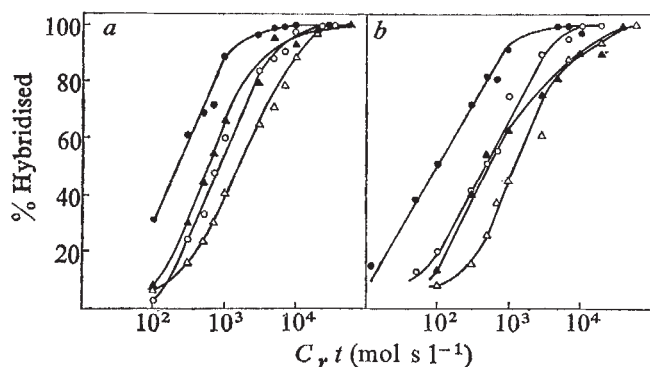


Fig. 3 Detection and measurement of sarcoma-specific RNA in the nucleus and cytoplasm of transformed and revertant field vole cells. Nuclear and cytoplasmic RNA from vole cells was prepared as follows. Frozen cell pellets were thawed rapidly at 37 °C and resuspended in RSB buffer (0.01 M Tris-HCl (pH 8.3), 0.01 M NaCl, 0.0015 M MgCl₂) at approximately 5×10^7 cells ml⁻¹. The cell suspension was adjusted to 0.5% Nonidet P-40, incubated at 4 °C for 5 min at which time cell disruption was completed by ten strokes of a Dounce homogeniser. The nuclei were pelleted by centrifugation at 700g for 10 min, washed once with RSB buffer and then several times with RSB buffer containing 1% Nonidet P-40 and 0.5% sodium deoxycholate. The nuclear washes were pooled with the cytoplasmic fraction which was further clarified by centrifugation at 13,000g for 5 min. The nuclei were lysed by resuspension in buffer containing 0.01 M Tris-HCl (pH 7.4), 0.5 M NaCl, 0.006 M MgCl₂, 0.6 mM CaCl₂ and adjusted to 50 µg of RNase-free pancreatic DNase ml⁻¹. After incubation at room temperature for 20 min, EDTA was added to 0.01 M. Nuclear and cytoplasmic fractions were then incubated for 30 min at 37 °C in the presence of 0.5% SDS and 500 µg of Pronase B (self-digested) ml⁻¹. The preparations were adjusted to 0.05 M sodium acetate (pH 5.1), 0.01 M EDTA and 1% SDS, extracted three times at 56 °C with an equal volume of phenol saturated with 0.05 M sodium acetate (pH 5.1), 0.01 M EDTA, and precipitated twice with ethanol. Nucleic acids were pelleted at 13,000g for 30 min, resuspended in DNase buffer and digested with RNase-free DNase as described above. The samples were re-extracted with phenol-chloroform-isoamyl alcohol and precipitated with ethanol as described for the preparation of total cellular RNA.

Contamination of cytoplasmic fractions by nuclear RNA was less than 1% as determined by previously published procedures²⁸. As far as cytoplasmic contamination of nuclear RNA was concerned, no obvious cytoplasmic material was present in the nuclei preparations as determined by contrast-phase microscopy. Furthermore, nuclear RNA preparations contained less than 5% 18S ribosomal RNA which is indicative of cytoplasmic contamination²⁹. Hybridisation of nuclear and cytoplasmic RNA to cDNA_{src} was performed at 68 °C for 70 h as described in Fig. 1. Each point represents an average of three separate determinations. *a*, Cytoplasmic RNA; *b*, nuclear RNA. ○, RSV-transformed field vole clone 1; △, RSV-transformed field vole clone 22; ▲, revertant field vole subclone 4; ●, revertant field vole subclone 866.

RSV-transforming gene sequences since little or no difference in either the amount or the extent of transcription can be detected in these two cell types. Since the viral transforming gene sequences are potentially active in the revertant cell these data suggest that the control of the transformed phenotype in this cell system is under the influence of some post-transcriptional control mechanism(s). The fact that no differences in the amount of sarcoma-specific RNA found associated with polyribosomes could be detected between the transformed and revertant cell type further indicates that this post-transcriptional regulation does not involve either restriction of transport of sarcoma-specific RNA from the nucleus to the cytoplasm, or the lack of formation of sarcoma-specific polyribosomes in the revertant cell type. Although previous studies on other transformed-revertant cell systems have indicated that regulation of expression of the sarcoma gene sequences can occur at the transcriptional level⁹⁻¹², the data presented here is the first indication that the expression of this particular gene sequence

can also be regulated post-transcriptionally. Thus the mere presence of sarcoma virus-specific RNA in eukaryotic cells may not be sufficient to initiate transformation following infection of cells by RNA tumour viruses since the host cell must play an important part in determining which particular gene sequences are ultimately expressed. This may explain the situation that exists in normal avian cells which contain low levels of sarcoma-specific RNA but are by no means transformed (D. Spector, H. Varmus & J. M. Bishop, personal communication) and may reflect the requirement of a host cell component that the sarcoma gene product must interact with in order to establish and/or maintain transformation. It is conceivable that this host cell component is absent in subclones of revertant vole cells. These transformed and revertant field vole cells should therefore provide us with a eukaryotic cell system with which to study the transformed phenotype and the epigenetic host factors that are operative and required either to regulate or maintain the expression of the viral transforming gene functions. Experiments are currently in progress to determine if viral-specific proteins, including the sarcoma gene product, can be detected in the revertant subclones. Retransformation of the revertant field vole cells by RSV is also being attempted. Collectively these studies should contribute to our understanding of the level of restriction of sarcoma

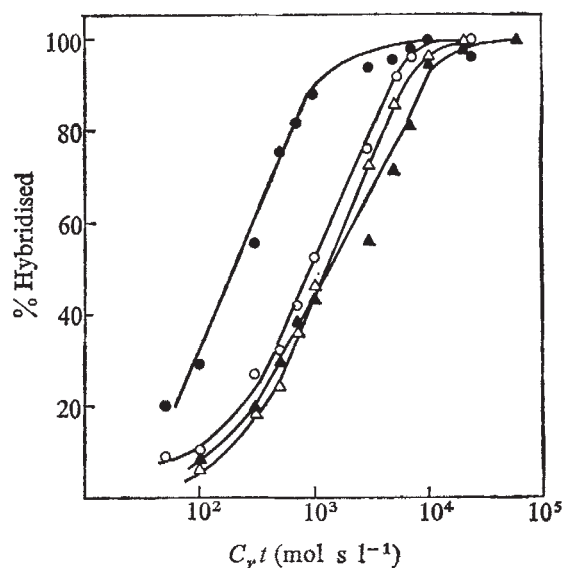


Fig. 4 Detection and measurement of polyribosome-associated sarcoma-specific RNA in transformed and revertant vole cells. Total polyribosomal RNA from transformed and revertant vole cells was prepared essentially according to the method of Gielkins *et al.*³⁰. Briefly, cells were washed with phosphate-buffered saline containing 200 µg of cycloheximide ml⁻¹, resuspended in buffer containing 0.05 M Tris-HCl (pH 8.5), 0.225 M KCl, 0.008 M MgCl₂, 0.002 M dithiothreitol, 500 µg of heparin, 200 µg of cycloheximide ml⁻¹ and 2% Nonidet P-40 and disrupted with a Dounce homogeniser. Nuclei and cell debris were removed by centrifugation and the 13,000g post-nuclear supernatant was layered on to a discontinuous sucrose gradient. Polyribosomes were pelleted by centrifugation, resuspended in 0.1 M Tris-HCl (pH 9.0) containing 0.5% SDS, extracted three times with phenol-chloroform-isoamyl alcohol (50:50:1) and precipitated twice with ethanol. Polyribosomal RNA was then digested with RNase-free DNase, re-extracted with phenol-chloroform-isoamyl alcohol and precipitated with ethanol as described in Fig. 1. In control experiments, treatment of the polyribosomal pellets with 30 mM EDTA for 15 min at room temperature released 90% of the sarcoma-specific RNA sequences consistent with its function as mRNA. Hybridisation of polyribosome-associated RNA to cDNA_{src} was performed for 70 h at 68 °C as described in Fig. 2. Each point represents an average of two to four separate determinations. ○, RSV-transformed field vole clone 1; △, RSV-transformed field vole clone 22; ▲, revertant field vole subclone 4; ●, revertant field vole subclone 866.

gene expression in this cell system and ultimately, elucidation of the precise nature of the cell function involved.

We thank S. Kanellos and D. Nelson for excellent technical assistance and L. Honza for typing this manuscript. We also thank Dr H. Varmus for invaluable discussions and communication of data before publication, and Drs J. Yunis and L. de la Mazafir for providing us with the normal vole cell line. We are indebted to Dr J. Gruber and the office of Program Resources and Logistics, Viral Cancer Program, Division of Cancer Cause and Prevention, National Cancer Institute for supplying us with valuable reagents. This investigation was conducted under Contract NO1-CP-61055 within the Virus Cancer Program of the National Cancer Institute. R. K. and A. L. are currently postdoctoral fellows supported by NIH postdoctoral fellowship CA 05231 and training grant CA 09 138 from the National Cancer Institute, respectively.

RICHARD A. KRZYZEK

ALAN F. LAU

ANTHONY J. FARAS

Department of Microbiology,
University of Minnesota Medical School,
Minneapolis, Minnesota 55455,

DEBORAH H. SPECTOR

Department of Microbiology,
University of California,
San Francisco, California 94122

Received 25 April; accepted 20 June 1977.

- ¹ Martin, G. S. *Nature* **227**, 1021–1023 (1970).
- ² Kawai, S. & Hanafusa, H. *Virology* **46**, 470–479 (1971).
- ³ Bader, J. P. *J. Virol.* **10**, 267–276 (1972).
- ⁴ Scolnick, E. M., Stephenson, J. R. & Aaronson, S. A. *J. Virol.* **10**, 653–657 (1972).
- ⁵ Graf, T. & Friis, R. R. *Virology* **56**, 369–374 (1973).
- ⁶ Wyke, J. A. & Linial, M. *Virology* **53**, 152–161 (1973).
- ⁷ Vogt, P. K. *Virology* **46**, 939–946 (1971).
- ⁸ Duesberg, P. H., Vogt, P. K., Maisel, J., Lai, M. & Canaani, E. *ICN-UCLA Symposium on Molecular Biology II*, 327–338 (Academic, New York, 1973).
- ⁹ Deng, C.-T., Boettiger, D., MacPherson, I. & Varmus, H. E. *Virology* **62**, 512–521 (1974).
- ¹⁰ Bishop, J. M., Deng, C.-T., Mahy, B. W. J., Quintrell, N., Stavnezer, E. & Varmus, H. E. *ICN-UCLA Symposia on Molecular and Cellular Biology*, IV, 1–20 (Academic, New York, 1976).
- ¹¹ Deng, C.-T., Stehelin, D., Bishop, J. M. & Varmus, H. E. *Virology* **76**, 313–330 (1977).
- ¹² Peebles, P. T., Scolnick, E. M. & Hawk, R. S. *Science* **192**, 1143–1145 (1976).
- ¹³ Toyoshima, K. & Vogt, P. K. *Virology* **39**, 930–931 (1969).
- ¹⁴ Friis, R. R., Toyoshima, K. & Vogt, P. K. *Virology* **43**, 375–389 (1971).
- ¹⁵ de la Maza, L. M., Faras, A. J., Varmus, H., Vogt, P. K. & Yunis, J. J. *exp. Cell Res.* **93**, 484–487 (1975).
- ¹⁶ Varmus, H. E., Levinson, W. E. & Bishop, J. M. *Nature new Biol.* **233**, 19–21 (1971).
- ¹⁷ Stehelin, D., Guntaka, R. V., Varmus, H. E. & Bishop, J. M. *J. molec. Biol.* **101**, 349–365 (1976).
- ¹⁸ Stehelin, D., Varmus, H. E., Bishop, J. M. & Vogt, P. K. *Nature* **260**, 170–173 (1976).
- ¹⁹ Collet, M. S., Kieras, R. M. & Faras, A. J. *Virology* **65**, 436–445 (1975).
- ²⁰ Leong, J.-A., Garapin, A.-C., Jackson, N., Fanshier, L., Levinson, W. E. & Bishop, J. M. *J. Virol.* **9**, 891–902 (1972).
- ²¹ Sutton, W. D. *Biochim. biophys. Acta* **240**, 522–531 (1971).
- ²² Haase, A., Traynor, B., Ventura, P. & Alling, D. *Virology* **70**, 65–72 (1976).
- ²³ Hillova, J., Mariage, R. & Hill, M. *Virology* **67**, 292–296 (1975).
- ²⁴ Graham, F. L. & Van Der Eb, A. J. *Virology* **52**, 456–476 (1973).
- ²⁵ Boettiger, D. *Virology* **62**, 522–529 (1974).
- ²⁶ Girard, M. *Methods in Enzymology*, **XIIA**, 581–588 (Academic, New York, 1967).
- ²⁷ Faras, A. J., Taylor, J. M., Levinson, W. E., Goodman, H. M. & Bishop, J. M. *J. molec. Biol.* **79**, 3874–3878 (1974).
- ²⁸ Varmus, H. E., Guntaka, R. V., Deng, C. T. & Bishop, J. M. *Cold Spring Harbor Symp. quant. Biol.* **39**, 3874–3878 (1974).
- ²⁹ Pezman, S. *J. molec. Biol.* **17**, 117–130 (1966).
- ³⁰ Gielkins, A. L. S., Salden, M. H. L. & Bloemendal, H. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1093–1097 (1974).

Cis-trans isomerisation in rhodopsin occurs in picoseconds

It has been believed for some time that the primary event in vision, the photochemical formation of bathorhodopsin, can be attributed to a *cis-trans* photoisomerisation¹. Recently this model has been questioned. Busch *et al.* proposed that the less-than-6-ps formation time of bathorhodopsin from rhodopsin does not allow significant isomerisation of the 11-*cis* chromophore to an all-*trans* isomer². This apparent difficulty with the *cis-trans* photoisomerisation model has prompted alternative models including (1) a mechanism involving deprotonation of the Schiff base nitrogen³, (2) proton transfer from the retinal methyl at position

five to opsin^{4,5} (involving the shifting of double bonds along the polyene chain to form a 'retro' type retinal), and (3) a photoinduced electron transfer to retinal from a protein donor group⁶. We have approached this question by performing picosecond absorption kinetic measurements on the formation time of bathorhodopsin from bovine rhodopsin and isorhodopsin. The essence of this experiment is that bathorhodopsin, being the common photo-product of rhodopsin (11-*cis* retinal) and isorhodopsin (9-*cis* retinal) must be an isomerised product of at least one of these pigments, but could be a product of both pigments (that is, basically all-*trans* retinal). Thus formation time measurements of bathorhodopsin from the two primary pigments can settle whether isomerisation can take place on the picosecond time scale.

Rhodopsin is the visual pigment in disk membranes of vertebrate rod cells⁷. It is composed of the chromophore, 11-*cis* retinal, covalently linked through a protonated Schiff base to a small protein called opsin. On absorption of a photon by the chromophore, a consecutive series of thermal intermediates is formed ending in the release of all *trans* retinal and free opsin^{8,9}. The opsin binding site also accommodates a 9-*cis* retinal. This pigment, isorhodopsin, has the same thermal intermediates as rhodopsin. The first thermal intermediate, bathorhodopsin, has been shown to be generated from rhodopsin in less than 6 ps at room temperature and thermally decays in about 100 ns (ref. 2).

The picosecond apparatus is detailed in Fig. 1. Generated pump and probe pulses were at 530 and 561 nm respectively. Rod outer segment membrane fragments were isolated from dark-adapted bovine retinae essentially as described previously¹⁰. Solubilised rhodopsin was obtained by extracting the membrane fragments in 3% lauryldimethylamine oxide and 50 mM phosphate buffer (pH 6.6). Isorhodopsin was prepared photochemically¹¹ by taking some of the rhodopsin samples to liquid nitrogen temperature, irradiating using the 568.2-nm krypton laser light, and warming. All measurements were taken at room temperature, and the sample stirred between each laser pulse to avoid light induced sample changes in the laser light path. No average sample degradation was observed during the experiments as measured by standard ultraviolet-visible absorption spectroscopy.

In these measurements, care must be taken so as not to over drive the sample in the laser beam during the picosecond pulse. For example, significant amounts of isorhodopsin can be produced from a rhodopsin sample at high light levels. Calculations using an average intensity for the laser pulse, the quantum yields^{12,13} and absorption constants at the pump wavelength show that less than 10% of the rhodopsin sample in the laser path would be converted to isorhodopsin and similarly for isorhodopsin to rhodopsin. Also, for the pump intensities used, the yield of bathorhodopsin starting with isorhodopsin would be about half the yield of bathorhodopsin from rhodopsin, in agreement with the experimental results.

The time dependence of the laser-induced absorbance changes for the conversion of rhodopsin and isorhodopsin to bathorhodopsin are shown in Fig. 2. The salient feature of the curves is the rapid rise within 9 ps. Thus, the photochemical formation of bathorhodopsin from either rhodopsin or isorhodopsin occurs in less than 9 ps. The time to reach half the limiting absorbance change¹⁴ is about 3 ps. This is an indication of the formation time of bathorhodopsin. The formation time from rhodopsin is in agreement with the work of Busch *et al.*².

As briefly argued above, bathorhodopsin must be an isomerised product of at least one of the primary pigments. Proof for this is given by recent resonance Raman work (a technique sensitive to conformation¹⁵). Similarities between the resonance Raman spectra of the 11-*cis* protonated Schiff base in solution and rhodopsin, and between the 9-*cis* protonated Schiff base and isorhodopsin, show that the conformations of the retinals of rhodopsin and isorhodopsin are 11-*cis* and 9-*cis* respectively, and are not particularly distorted by the surrounding protein^{16,17}. In addition, the resonance Raman spectrum of photochemically produced isorhodopsin¹¹ is identical to that of regenerated 9-*cis* retinal¹⁸ and opsin, proving that complete photoisomerisation(s) about two double bonds takes place in the rhodopsin→bathorhodopsin→isorhodopsin phototransitions. Either at the

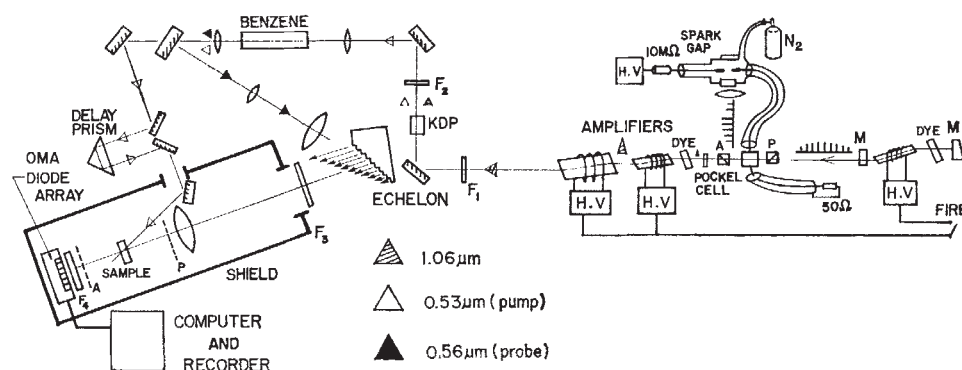


Fig. 1 Schematic representation of the experimental system. The components include: a Nd^{3+} glass oscillator with cavity mirrors M and saturable dye absorber; a single pulse selector consisting of two crossed polarisers, Pockels cell and spark gap; a half wave plate and 'clean-up' saturable dye absorber; two Nd^{3+} glass amplifiers (gain $\times 20$); filter F_1 to eliminate flash lamp; a second harmonic generator; filter F_2 to eliminate $1.06 \mu\text{m}$; a 15-cm benzene cell to produce stimulated Raman scattering at 561 nm ; specially coated mirrors to separate the 530 nm and 561 nm beam paths; in the 561 nm path, an expand-

ing telescope lens system, a time delayed reflection echelon (14 steps at 4.5 ps per step), filters F_3 and F_4 to eliminate 530 nm , a focusing lens on sample and an optical multichannel analyser detector system; in the 530 nm path, a delay prism and directing mirrors. The full width at half maximum for the $1.06 \mu\text{m}$ and 530 nm pulses are 9 ps and 7 ps respectively.

first or the second photoreaction or at both photoreactions a major isomerisation must take place. Thus, there is no doubt that bathorhodopsin, being a common photoproduct of both rhodopsin and isorhodopsin, is an isomerised product of at least one of the primary pigments.

Our picosecond results show that major photo-induced isomerisation(s) of retinal in bovine rhodopsin can take place on a picosecond time scale since both rhodopsin $\rightarrow$ bathorhodopsin and isorhodopsin $\rightarrow$ bathorhodopsin photoreactions take place in less than 9 ps . In agreement with our observations, Warshel¹⁹ has shown that picosecond isomerisation times are theoretically feasible in rhodopsin, although this theoretical approach relies heavily on the exact nature of the excited state potential surfaces which are difficult to obtain precisely.

Huppert *et al.*⁹ have performed kinetic measurements with protonated retinal Schiff bases in solution, and report nanosecond recovery kinetics which are ascribed to isomerisation times. From our results and theirs, it seems likely that in the visual pigments the photoisomerisation times are greatly accelerated by chromophore protein interactions. That the protein significantly

modifies this retinal property is not surprising. In contrast to retinals in the visual pigments, protonated retinal Schiff bases in solution have blue-shifted absorption bands and much reduced, wavelength dependent quantum yields of photoisomerisation¹³.

Finally, our results do not describe the exact nature of bathorhodopsin. Rosenfeld *et al.*^{1,3}, however, using the basic early arguments of Hubbard and Kropf¹ and Yoshizawa and Wald²⁰ concerning the properties of bathorhodopsin (or lumirhodopsin), recent resonance Raman data¹⁵, and recent artificial pigment work have recently argued that the chromophore of bathorhodopsin must have essentially a *trans* conformation but not necessarily a planar one. Our results remove any uncertainty from these arguments due to the picosecond isomerisation times.

We thank Professor Honig for useful discussions, and Princeton Applied Research for the use of an OMA. This work was supported in part by US NSF grants (P C M 75 13155A01 and BMS 75-03020) and grants from City University Research Award Programs and Philips Laboratories. B.H.G. is a Philips Fellow and R.R.A. an Alfred P. Sloan Fellow.

B. H. GREEN

T. G. MONGER

R. R. ALFANO

B. ATON

R. H. CALLENDER

Department of Physics,
The City College of The City University of New York,
New York, New York 10031

Received 19 April; accepted 5 July 1977.

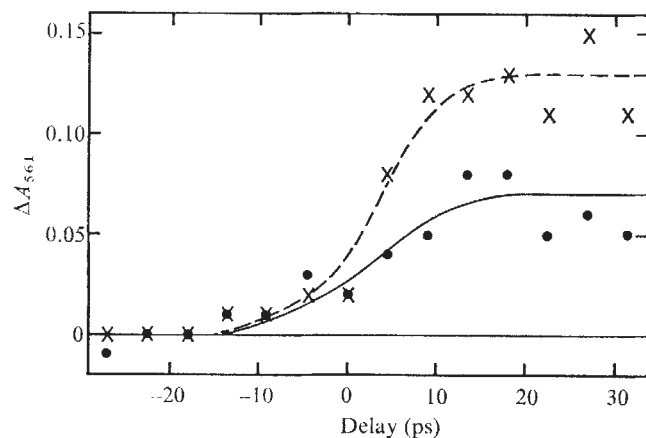


Fig. 2 Laser-induced absorbance changes at 561 nm as a function of time in detergent solubilised bovine rhodopsin ($\times$) and isorhodopsin ($\bullet$) at room temperature. Bathorhodopsin is the only intermediate during the bleaching of bovine rhodopsin known to absorb strongly at 561 nm . The energy of the 530 nm pump pulse was about 10^{-4} J ; the energy of the 561 nm probe pulse was about 10^{-7} J . The beam sizes were about 1 mm^2 for the pump and 0.5 mm^2 for the probe. The samples (about 1.5 ml) were held in 0.5-cm cuvettes. The concentrations were about 4 A cm^{-1} at the absorption peaks near 500 nm ; the ratios A_{400}/A_{500} were about 0.3 ; and ratios A_{530}/A_{500} were about 0.7 for rhodopsin and 0.5 for isorhodopsin. Each data point shown is the average of six (rhodopsin) and nine (isorhodopsin) laser shots. Typical mean standard deviations are ± 0.03 . The zero time is located using a 0.5 cm CS_2 Kerr optical shutter²¹ at the sample site. The half width at half maximum for the CS_2 shutter prompt response curve is about 6 ps .

¹ Hubbard, R. & Kropf, A. *Proc. natn. Acad. Sci. U.S.A.* **44**, 130-139 (1958).

² Busch, G. E., Applebury, M. L., Lamola, A. A. & Rentzepis, P. M. *Proc. natn. Acad. Sci. U.S.A.* **69**, 2802-2806 (1972).

³ Thomson, A. J. *Nature* **254**, 178-179 (1975).

⁴ Fransen, M. R. *et al.* *Nature* **260**, 726-727 (1976).

⁵ van der Meer, K., Mulder, J. J. C. & Lugtenburg, J. *Photochem. Photobiol.* **24**, 363-367 (1976).

⁶ Huppert, D., Rentzepis, P. M. & Kliger, D. S. *Photochem. Photobiol.* **25**, 193-197 (1977).

⁷ Wald, G. *Science* **162**, 230-239 (1968).

⁸ Ebrey, T. & Honig, B. *Q. Rev. Biophys.* **8**, 124-184 (1975).

⁹ Honig, B. & Ebrey, T. *Rev. Biophys. Bioeng.* **3**, 151-177 (1974).

¹⁰ Hong, K. & Hubbell, W. L. *Biochemistry* **12**, 4517-4523 (1973).

¹¹ Oseroff, A. R. & Callender, R. H. *Biochemistry* **13**, 4243-4248 (1974).

¹² Dartnall, H. J. A. in *Handbook of Sensory Physiology*, V11, 1, 122-145 (1972).

¹³ Rosenfeld, T., Honig, B., Ottolenghi, M., Hurley, J. & Ebrey, T. G. *Pure appl. Chem.* **49**, 341-351 (1977).

¹⁴ Ricard, D., Lowdermilk, W. H. & Ducuing, J. *Chem. Phys. Lett.* **16**, 617-621 (1972).

¹⁵ Callender, R. H. & Honig, B. *Rev. Biophys. Bioeng.* **6**, 33-55 (1977).

¹⁶ Mathies, R., Oseroff, A. R., Freedman, T. B. & Stryer, L. in *Tunable Laser Applications* (eds Jaeger, T., Stokseth, P. & Mooradian, A.) (Springer, New York, in the press).

¹⁷ Mathies, R., Freedman, T. B. & Stryer, L. *molec. Biol.* **109**, 367-372 (1977).

¹⁸ Mathies, R., Oseroff, A. R. & Stryer, L. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1-5 (1976).

¹⁹ Warshel, A. *Nature* **260**, 679-683 (1976).

²⁰ Yoshizawa, T. & Wald, G. *Nature* **197**, 1279-1286 (1963).

²¹ Duguay, M. A. & Hansen, J. W. *Appl. Phys. Lett.* **15**, 192-194 (1969).

reviews

Darwin's papers

Frederick Burkhardt

The Collected Papers of Charles Darwin. (2 Volumes.) Edited by Paul H. Barrett, with a Foreword by Theodosius Dobzhansky. Pp. viii+277+326. (University of Chicago: Chicago and London, 1977.) £27.30; \$40 the set.

THESE volumes bring together 152 papers which Darwin published over a period of forty-seven years. They range in length from a paragraph to forty-eight pages. Thirty-three of them first appeared in *Nature*, between 1869 and 1882. Others were contributions to learned societies of which Darwin was a member: the Geological (12), Linnean (9), Zoological (4), Geographical (1), and the Royal (1). (His sole contribution to the Royal Society was his solution of the problem of the 'Parallel Roads of Glen Roy', a paper he later called a 'gigantic blunder'.)

The largest number of items (50) are from *The Gardeners' Chronicle and Agricultural Gazette*, the medium Darwin used for communication with plant and animal breeders. The rest of the papers are mainly from periodicals concerned with popular but serious discussion of scientific matters, like the *Natural History Review*.

An appendix lists fifty-two articles dealing with the species collected by Darwin on the *Beagle* voyage, in itself a considerable bibliographical contribution. All of Darwin's notes are preserved. Professor Barrett's notes are relatively few in number and are concerned with identifying persons and places and explaining technical terms.

The effect produced by the papers in their totality is dramatic in the way they make clear the range of Darwin's interests, his powers of observation, his imaginative questioning, his probing for facts that he hoped would corroborate his hypotheses, and his astonishing talent for experimentation.

The papers naturally vary considerably in scientific and historical significance. Some are famous, like the joint paper with Wallace in which Natural Selection was first announced, and his remarkable papers on plant physiology.

Some of the brief and apparently ephemeral items are now seen to be important in Darwin's work on his species theory. As Theodosius Dobzhansky points out in his appreciative and graceful Foreword, Darwin's short query about 'A Mouse-Coloured Breed

of Ponies' was really asking 'Have the species horse and ass descended from a common ancestor'?. Similarly, Darwin's experiments on the ability of seeds to survive immersion in salt water were designed to provide him with an explanation of the geographical distribution of species that could dispense with creationism and Forbes's vast continental extensions.

Although this is primarily a reference work, there is plenty of interesting and readable material in it. Darwin wrote many of the pieces in clear non-technical prose for those interested in such subjects as erratic boulders, the fine dust that falls on ships in the Atlantic, mimetic butterflies, the fertilisation of British orchids, the origin of certain instincts, or the biographical sketch of an infant.

Scholars will want to know how complete the collection is. The answer depends on what criteria are used in selection. Professor Barrett includes only papers published by Darwin in his lifetime. Richard Freeman, in his new edition of the bibliographical handlist of Darwin's works includes posthumous articles, memorials, and items privately printed and circulated by Darwin. On

Professor Barrett's criteria, each work has four items overlooked by the other. Using Mr Freeman's standards, but omitting repetitive printings, nine more might have been included. For example, Darwin's important 'Queries about the Breeding of Animals' is omitted by Barrett because it was printed and distributed privately, but the 'Queries about Expression' is included because it found its way into an Annual Report of the Smithsonian Institution. Again, Professor Barrett omits three items because the books to which they were contributed were published after Darwin's death. Since these circumstances in no way affect the authenticity of the writings or their importance, it seems arbitrary to deny them a place in a *Collected Papers*.

This editorial question aside, Professor Barrett deserves great credit for making these writings available in a convenient and reliable collection in which scholars and non-specialists alike will find both profit and pleasure.

Frederick Burkhardt, President Emeritus of the American Council of Learned Societies, is co-editor, with Sydney Smith, of the prospective Collected Letters of Charles Darwin.

Complex systems

The Foundations of Cybernetics. By F. H. George. Pp. xiv+286. (Gordon and Breach: London, Paris and New York, 1977.) £13.50.

THE last page of this book contains the following comment "... about the word Cybernetics. In America it is now slightly frowned upon, and although the word is currently more acceptable in the United Kingdom it is still suspect in some quarters. . . . The problem stems from the arbitrary divisions we have in our sciences, and the very slow conservative attitude of scientists towards the development of scientific ideas and classifications. . . ."

Up to a point the complaint deserves sympathy. Much can be gained from interdisciplinary work. There is, of course, little to stop the individual from drawing his own "arbitrary divisions". Whether his new subject becomes acceptable to the larger community depends on what it has to offer and the following it can attract.

Broadly, Cybernetics encompasses the study of complex systems. On the one hand there are the man-made systems of automation and computer technology and, on the other, those provided by nature, such as the brain of man and other physiological organs. There is, one hopes, a conceptual framework common to both. The man-made systems can serve as a source of analogies for the development of theory in psychology and physiology while, in the reverse direction, the living world can act as a source of inspiration to inventors.

The book is concerned with the, as yet undeveloped and unconnected, conceptual framework that may serve the two sides. There are chapters on feedback, finite automata, neural nets, logic, computers, information theory, and aspects of psychology and physiology. With a net cast so wide, it is not surprising to find that the treatment of some topics is superficial. This means that for information on any one

of the topics one could do better by turning to more specialised sources. As a book of its kind, however, it is one of the best.

It is clear from the evidence presented that Cybernetics still awaits its breakthroughs. As a body of theory it has little to offer and seems to have taken almost everything it has from other disciplines. The developments envisaged by Wiener, Ashby, and others, that can result from the interaction of ideas from biology and technology do, of course, continue to take

place; but, perhaps not, as they had hoped, under the heading of Cybernetics. Ironically, there is the consolation that the "arbitrary divisions" which, some thirty years ago, marked out this new field of science are not likely to become hard-set, and thereby to obstruct future changes in the internal boundaries of science.

V. Zakian

V. Zakian is Lecturer at the University of Manchester Institute of Science and Technology, Manchester, UK.

Heterocyclic chemistry

Stereochemistry of Heterocyclic Compounds. Part 2: Oxygen, Sulfur, Mixed N,O, and S, and Phosphorus Heterocycles. By W. L. F. Armarego. Pp. xviii + 494. (Wiley-Interscience: London and New York, 1977.) £32.65; \$54.

ALTHOUGH the literature of organic chemistry includes many comprehensive surveys of the stereochemistry and conformational analysis of carbocyclic compounds, scant attention has hitherto been given to the corresponding aspects of heterocyclic chemistry. Numerous discussions of isolated topics in this vast, comparatively neglected area are scattered throughout the review literature, but the present two-volume set appears to be the first monograph to attempt a review of this field as a whole. This second volume is devoted to the stereochemistry of oxygen, sulphur, mixed nitrogen, oxygen and sulphur heterocycles, and a final chapter deals with phosphorus heterocycles.

The organisation of the volume is the same as that of the first, even to the extent that the first brief, introductory chapter, which explains the order of discussion of the data, is identical in the two volumes. Each section begins with a discussion of the stereochemical aspects of the synthesis of compounds in the group, then follows a discussion of the configuration, and then the conformation, of the compounds, and finally stereochemical aspects of their reactions are discussed. The whole project is one of daunting proportions; this is amply illustrated by the fact that this second volume alone contains a bibliography of over 2,400 references, which cover the subject completely up to the end of 1974, and incompletely for 1975. Presumably because of the enormous volume of material to be reviewed, the final chapter has been contributed by M. J. Gallagher.

The coverage of this material in under 450 pages of text has resulted in inevitable restrictions; the style is of

necessity compressed, but it is clear, critical, and very readable. Where earlier authoritative reviews exist, the author has been concerned primarily with updating them. Important stereochemical principles and illustrations of syntheses and reactions are taken mainly from the behaviour of model compounds, and a discussion of the vast number of natural products is not attempted. In the present context this includes, for example, furanose and pyranose carbohydrates, penicillins and cephalosporins. Steroidal epoxides are also among the groups omitted; however, in all these areas, the excellent bibliography provides a ready entry into these aspects of the subject, as well as providing a comprehensive bibliography for readers who wish to pursue the subject matter of the text in greater depth.

The book is beautifully produced, and typographical errors are rare. The formulae have been very skilfully drawn by the author's talented amanuensis, the heteroatoms and substituent groups being inserted manually rather than in the usual type-face. This has presumably resulted in some economy in production, and it has also obviated one possible source of errors, without any sacrifice of production standards. There is a moderately detailed subject index, which also includes the names of authors mentioned in the text; there is no separate, complete, author index.

This volume certainly fills a very important gap in the review literature of heterocyclic chemistry, and since, with its companion volume on nitrogen heterocycles, it heralds the introduction of a new series of monographs on general heterocyclic chemistry, each volume in which will survey one aspect of the *whole field* of heterocyclic chemistry, it is to be particularly welcomed. It will be immensely useful to advanced undergraduates and to research workers in heterocyclic chemistry, and it surely belongs in every chemistry library and heterocyclic research laboratory.

J. E. Saxton

J. E. Saxton is Senior Lecturer in Organic Chemistry at the University of Leeds, UK.

Biological tools

Introduction to the Spectroscopy of Biological Polymers. Edited by D. W. Jones. Pp. xii + 328. (Academic: London and New York, 1977.) £11.60; \$25.50.

SPECTROSCOPIC techniques have played no less an important part in the structural studies of biological macromolecules than they have in more classical studies of simpler molecules, and the range of spectroscopies, if differing in emphasis, has been equally as wide. Consequently, an author or editor aiming to produce a book that covers all or most of the spectroscopic techniques used in studying biological polymers sets himself an unenviable task. He not only attempts a synthesis of several highly specialised subjects but does so, unlike his many predecessors who have undertaken similar enterprises in a purely chemical context, knowing he can make very few assumptions about his readers' knowledge of mathematics and physics.

The editor of this work has overcome the problem of the multidisciplinary nature of his subject by choosing an author eminent in each field to contribute a chapter on that speciality. He has sandwiched these chapters between a general introduction to molecular spectroscopy and a concluding chapter briefly describing some examples that illustrate the power of combining several spectroscopic techniques to tackle a specific biological problem. The approach throughout is descriptive and not mathematical, and several difficult physical concepts are explained clearly.

The most original aspect of the book is the strong emphasis placed on the use of vibrational spectroscopy. The chapter on Raman spectroscopy is particularly welcome as advances in laser technology over the past decade have made this technique a powerful biological tool. No less welcome and more surprising are the two chapters on infrared spectroscopy. They illustrate the potential of this technique especially when combined with Fourier-transform analysis. They also link the work done on biological polymers with that done on the industrially important synthetic polymers. The remaining chapters cover absorption, emission, optical rotation, circular dichroism, nuclear magnetic resonance, electron spin resonance and Mössbauer spectroscopy.

Michael T. Flanagan

M. T. Flanagan, until recently a member of the Biophysics Division of the National Institute of Medical Research, London, is now with Standard Telecommunication Laboratories, Harlow, UK.

Space-time proceedings

Asymptotic Structure of Space-Time. Edited by F. Paul Esposito and Louis Witten. Pp. vii+442. (Plenum: London and New York, 1977.) \$51.

IN recent years it has increasingly become the practice for the proceedings of international scientific conferences to be published a year or so later in a single volume at an exorbitant price. Occasionally these collected works are very useful as a comprehensive review of a new or rapidly developing subject, and if well written and edited can provide students with a convenient access to up-to-date work. All too often, however, the proceedings are a hotch-potch of incomplete articles, bearing only a superficial connection to each other, and coexisting under the same cover merely to avoid offending some of the conference contributors.

The usefulness of conference proceedings is still further reduced when preprints of the papers are thoroughly circulated beforehand and all the publisher does is to bind together reproductions of these manuscripts (often containing barely legible hand-written mathematical symbols and a multiplicity of type faces) and offer the end-product for anything up to a few dozen dollars.

Many of these volumes are now being offered to university libraries labouring under stringent budget controls, so that would-be purchasers are becoming much more discerning in their requirements. They will be interested to know that *Asymptotic Structure of Space-Time* is, by these recent standards, very well put together. True, the price, at \$51, is regrettably high, but most of the manuscripts have been well prepared and typed, and the papers are of a fairly uniform style and standard. The conference took place in Cincinnati in June 1976, and covers a selection of important and fast-moving new developments in mathematical relativity and space-time structure. After a useful and well written introduction by R. Geroch the reader is treated to a discourse on cone space, (Brian Bramson), conformal bundle boundaries (B. G. Schmidt) and complex vacuum metrics (J. F. Plebanski and I. Robinson). I was disappointed that the theory of twistors did not receive some prominence, but there are two good papers on the related topic of H-space (E. T. Newman, K. P. Tod and M. Ko), although these are far too condensed.

The recent resurgence of interest in quantum aspects of gravitation is reflected in a (once again, too short)

paper on supersymmetry by P. van Nieuwenhuizen, together with an excellent and very readable review on quantum field theory in curved space and particle production by gravitational fields, by Leonard Parker. I found this one of the best presented of all the papers, and its list of 168 references is a masterpiece of literature research.

My overall impression of this book is that it is well edited and presented, and is nice to have available.

Paul Davies

Paul Davies is Lecturer in Mathematics at King's College, University of London, UK.

Methylmercury intoxication

Minamata Disease: Methylmercury Poisoning in Minamata and Niigata, Japan. Edited by T. Tsubaki and K. Irukayama. Pp. 310. (Kodansha: Tokyo; Elsevier: Amsterdam, New York and Oxford, 1977.) \$48.25; Dfl. 118.

METHYLMERCURY intoxication is now frequently called Minamata disease as it was first around the Minamata Bay that methylmercury caused a mass epidemic. One of the best parts of the book deals with the background and epidemiology of this and a similar epidemic in Niigata. The Minamata epidemic started in 1941 with individual cases and its epidemic proportion was noticed in 1956 when it was first believed to be of infectious origin. In 1958 Dr McAlpine, who saw some of the patients, called attention to the similarity between Minamata Disease and methylmercury intoxication. His paper written with Dr Araki and published in the *Lancet* in 1958, but not mentioned in the otherwise detailed historical and clinical account, turned the attention to mercury; and subsequently methylmercury was identified in sediments, seafood and in the drainage system of the Minamata factory.

Although the number of yearly cases declined, new cases were reported as late as 1972-73, which is not surprising as the mercury concentration in shellfish remained up to that period of about 5 p.p.m., but sometimes as high as 16 p.p.m. The situation was not very different in Niigata, where the ban on fishing in the Agano river did not prevent new outbreaks, which are discussed in this book as intoxications with delayed onset. Whether methylmercury is able to precipitate disease long after the cessation of exposure is an important problem, but inadequately handled in the whole book. There is also a certain reluctance to re-value past views: data based on the dithizone

method is interpreted without reservation although according to a table this method, compared with atomic absorption, generally underestimated tissue mercury levels by a factor of 3.

Translation is very uneven and often puzzling. A sentence like "After an interval of 14 years, one of the 4 cases came to necropsy, and was found to be grossly ataxic; blindness of the left eye and severe constriction of the visual field in the right eye persisted" is more than a puzzle, but it might illustrate the difficulty of interpreting some paragraphs with less blatant error. It might be that the translation is responsible for the surprising statement that mercury half-time in the brain was calculated from autopsy material.

Despite shortcomings, *Minamata Disease* is an important publication on the origin, epidemiology, pathology and clinical aspects of the two outbreaks in Japan. It would be a very much better book, however, if publications of this type were submitted to the same rules as any publication in a learned journal: revision based on the suggestions and criticisms of two referees.

Laszlo Magos

Laszlo Magos is Senior Scientific Officer at the MRC Toxicology Unit, Carshalton, UK.

BOOKS

ON PURE
AND APPLIED SCIENCE

Books reviewed or mentioned in this journal are available from stock.

Catalogues on application.

Please state interests.

SCIENTIFIC LIBRARY

ANNUAL SUBSCRIPTION from £7.00

Reduced rates for multiple subscriptions

Available in U.K. only

Prospectus free on request

H. K. LEWIS & Co. Ltd.

LONDON: 136 GOWER STREET,
WC1E 6BS

Telephone: 01-387 4282

Circle No. 08 on Reader Enquiry Card.

Functional groups

Chemistry of Functional Groups. Supplement A: Chemistry of Double-Bonded Functional Groups. Parts 1 and 2. Edited by S. Patai. Pp. 651 each part. (Wiley: London, New York Sydney and Toronto, 1977.) Part 1 £29; \$52.50; Part 2 £29.50; \$55. Suppl. as a set £58.50; \$107.50.

SUPPLEMENT A to *Chemistry of Functional Groups* is the first in a set of planned supplementary volumes to this well established series. The aim of the Editor is to include in them the "missing chapters" from earlier volumes in addition to providing other chapters to extend and bring up to date the material in the earlier volumes. The two parts of this supplement A relate chiefly to earlier volumes in the series on alkenes (1964 and 1970), the carbonyl group (1966), the carbon-nitrogen double bond (1970), and the azo group (1975).

The first part contains chapters in which all of the above groups are considered, usually separately within each chapter. Reviews on dipole moments, configurations and conformations of molecules, liquid crystals, and thermochemistry provide a timely reminder of the value of an understanding of the physical properties and structures of organic molecules as a basis for a study of their reactions and reactivity. The chapters which follow on the mechanisms of elimination and addition reactions, electrochemistry, 1,3-dipolar cycloadditions, and the reactions of carbenes, demonstrate the rapid developments in these areas in recent years, and provide the reader with a clear picture of the advances that have taken place.

Part two commences with a review on the formation of unsaturated groups by heterolytic fragmentation. It is particularly useful in showing that numerous apparently different reactions conform to a common pattern of reactivity and lead to the formation of compounds with double-bonded functional groups. The remaining chapters are on more specific subjects, and are concerned with reviews of electrophilic additions to carbon-carbon double bonds, the olefin metathesis reaction, oxidation of $-C=C-$ and $-C=N$ groups, and transition metal-catalysed carbonylation of olefins, imidines and diamides (1,3,5-triazapentadienes).

All chapters in the supplement are of the nature of essay reviews not claiming to give encyclopaedic coverage. They are well written with clear diagrams and reaction schemes. Although

coverage is selective, the authors have been careful to choose illustrative examples that demonstrate the essential principles they wish to convey. Cross references are given to earlier volumes in the series as well as to other reviews and to many individual papers. In some chapters as many as 500 references are provided.

The main value of this series is that a reader on studying a particular chapter will get a clear overall picture of the aspect of chemistry that it covers, and will be able to explore more deeply by consulting the references provided. In this supplement, the chapters vary in the ground they attempt to cover; some survey enormous fields, others bring together reactions of a number of different functional groups within a coherent framework, and yet others cover very specific topics. Within the framework of the

series these two volumes represent valuable additions to their parent volumes published earlier. They will be useful to undergraduate and postgraduate students, and also to research workers in industry. The scope of the series makes its price beyond the pockets of individuals but these volumes, like the others in the series, will be most important additions to the libraries of teaching and research establishments specialising in organic chemistry. The series is likely to increase in value particularly when, in these inflationary days, the range of review journals and periodical reviews subscribed to by libraries have to be curtailed on financial grounds.

D. I. Davies

D. I. Davies is Reader in Organic Chemistry at King's College, University of London, UK.

Water and tropical agriculture

Climate, Water and Agriculture in the Tropics. By I. J. Jackson. Pp. xii+248. (Longman: London and New York; 1977.) £3.75.

SINCE temperature is always adequate for plant growth in the tropics, the main climatological limitation to agriculture is often inadequate rainfall. *Climate, Water and Agriculture in the Tropics* by Jackson therefore covers what is potentially an important field. The author has spent six years lecturing in Tanzania, so he has first-hand experience of the problems and is well qualified to write this particular book. He examines the characteristics of tropical rainfall and evaporation, together with their implications, especially as related to agriculture, land use and other aspects such as soil erosion and irrigation. Early chapters consider the origins of tropical precipitation, rainfall seasonality and variability, and rainfall intensity. The general atmospheric circulation of the tropics is discussed briefly. Only one chapter is devoted to evaporation, with a short discussion of methods of estimation. The final chapters are concerned with water-plant relationships and the book ends with a discussion of man's impact on the hydrological cycle.

The tropics are vast in area, and cover a great variety of climatic types. It is therefore impossible in a book of limited length to cover all aspects of tropical plant-water relationships. Although the coverage of the book is

wide, it does tend to concentrate on the humid tropics of Africa and Asia, with particular emphasis on the statistical aspects of rainfall. Considering its importance to the energy balance of tropical plants, evaporation is discussed somewhat briefly. Evaporation depends largely on the available radiation, but there is no discussion of the distribution of solar radiation in the tropical world. Given an adequate water supply, plant yields depend to some degree on the available solar radiation, which in the summer hemisphere can increase away from the equator, leading to the highest crop yields in the subtropics. This is another reason for introducing some discussion on tropical solar radiation patterns. Considering that the book is essentially about water and tropical agriculture, it is a pity that there is not a really substantial section on the problems of drought and desertification in the tropics. Both problems have assumed some importance in recent years, especially as there are suggestions that there is a feedback mechanism between desert formation by overgrazing, and decreasing rainfall.

The main strength of this book lies in its discussion of tropical precipitation; here it fills a gap in the available literature on this subject. The book is well produced and well illustrated, and includes an extensive list of references. It should prove invaluable to specialists concerned with tropical water resources, and also provide useful reading for students of the agriculture, climatology or geography of the tropics.

J. G. Lockwood

J. G. Lockwood is Senior Lecturer in the School of Geography at the University of Leeds, UK.